BIOLOGICAL HYDROGEN PRODUCTION USING CO-CULTURES OF PNS BACTERIA IN AN IMMOBILIZED SETTING

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ABSTRACT

BIOLOGICAL HYDROGEN PRODUCTION USING CO-CULTURES OF PNS BACTERIA IN AN IMMOBILIZED SETTING

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Biological hydrogen production using purple nonsulfur bacteria is a promising field that would provide biofuel production from solar energy. This study focused on hydrogen production using co-cultures of different PNSB strains in an agar-immobilized setting and searched for both the synergistic effect of using co-cultures of PNSB and the potential advantages of utilizing an immobilized system.

Different bioreactors having volumes of 1.4 L, 300 mL and 150 mL have been tested. The photobioreactors were operated anaerobically indoor (continuous illumination) with different light intensities, such as 2500 lux and 4000 lux, and outdoors for 15-25 days using a defined H_2 production medium with different substrate concentrations, such as 40 mM Acetate + 2 mM Glutamate and 60 mM Acetate + 2 mM Glutamate. Best results were obtained by agar-immobilization of different PNSB strains, *R. capsulatus* hup- (YO3), *R. palustris* (DSMZ 127), and *R. sphaeroides* O.U.001 (DSMZ 5864) as single, double, and triple co-cultures on the inner surface of a glass 150 mL cylindrical bioreactor as biofilm formation.

The growth, hydrogen production capacities, substrate utilization, and pH changes were recorded.

The hydrogen yields were within 0.021-3.655 mol H_2/mol Acetate and H_2 productivities were within 0.005-0.737 mol H_2/L.h.

It was concluded that co-cultivation of PNSB did not result in higher H_2 yield and productivity compared to single cultures. However, the di-culture of *R. capsulatus* hup- (YO3) and *R. palustris* (DSMZ 127) seems as a promising co-culture couple, since together they perform optimal hydrogen production both in low and high substrate concentrations and light intensities, and also, H_2 production by immobilized PNSB in a biofilm format cylindrical reactor seems promising for large-scale H_2 output in outdoor photobioreactors since optimum illumination can be maintained despite the sun's varying incidence angles throughout the day and the year.

Keywords: Purple Nonsulfur Bacteria, Co-culture, Immobilization, Biological Hydrogen Production, Photofermentation

ÖZ

İMMOBİLİZE BİR YERLEŞİMDE MOR KÜKÜRTSÜZ BAKTERİLERİN ORTAK KÜLTÜRLERİ KULLANILARAK BİYOLOJİK HİDROJEN ÜRETİMİ

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Mor, kükürtsüz bakterilerle biyolojik H_2 üretimi, güneş enerjisinden biyoyakıt üretimi sağlayabilecek, umut veren bir alandır. Bu çalışma, agarla immobilize bir sistemde farklı PNSB suşlarının ortak kültürleri kullanılarak H_2 üretimine odaklanmış ve PNSB ortak kültürlerinin kullanılmasının sinerjik etkilerini ve immobilize bir sistem kullanmanın olası faydalarını bulmaya çalışmıştır.

1.4 L, 300 mL ve 150 mL'lik hacimlere sahip farklı biyoreaktörler denenmiştir. Fotobiyoreaktörler, 2500 ve 4000 lux gibi farklı ışık şiddetleriyle kaplı mekan (sürekli aydınlatma) ve açık hava koşullarında 15-25 gün boyunca 40 mM Asetat + 2 mM Glutamat ve 60 mM Asetat + 2 mM Glutamat gibi farklı substrat derişimlerine sahip tanımlı H_2 üretim besi yeri kullanılarak anaerobik olarak çalıştırılmıştır. En iyi sonuçlar, farklı PNSB suşları olan *R. capsulatus* hup- (YO3), *R. palustris* (DSMZ 127) ve *R. sphaeroides* O.U.001 (DSMZ 5864)'nin, 150 mL'lik bir cam silindir biyoreaktörün iç yüzeyine, biyofilm oluşumu şeklinde tekli kültürler, ikili ve üçlü ortak kültürler olarak agarla immobilize edilmesiyle elde edilmiştir.

Büyümeleri, H_2 üretim kapasiteleri, substrat kullanımları ve pH değişiklikleri kaydedilmiştir.

H_2 verimlerinin, 0.021-3.655 mol H_2/mol Asetat aralığında ve H_2 üretkenliklerinin, 0.005-0.737 mol H_2/L.h aralığında olduğu bulunmuştur.

Her ne kadar PNSB ortak kültürleri, tekli kültürlere göre daha yüksek H_2 verim ve üretkenlikleriyle sonuçlanmış olmasa da, *R. capsulatus* hup- (YO3) and *R. palustris* (DSMZ 127) ikili kültürü, birlikte hem düşük, hem de yüksek substrat derişimleri ve ışık şiddetlerinde optimal H_2 üretimi gerçekleştirdikleri için, umut veren bir ortak kültür ikilisi olarak görünmektedir ve ayrıca, biyofilm formatlı bir silindir reaktörde immobilize edilmiş PNSB vasıtasıyla H_2 üretiminin, güneşin gün ve yıl boyunca değişen geliş açılarına rağmen optimum aydınlanma sağlanabildiği için, açık hava fotobiyoreaktörlerinde büyük ölçekli H_2 üretimi için umut verdiği görülmektedir.

Anahtar Kelimeler: Mor Kükürtsüz Bakteriler, Ortak kültür, İmmobilizasyon, Biyolojik Hidrojen Üretimi, Fotofermentasyon

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ABBREVIATIONS	
ADP	Adenosine Di-Phosphate
ATP	Adenosine Tri-Phosphate
COD	Chemical Oxygen Demand
C. saccharolyticus	*Caldicellulosiruptor saccharolyticus*
Fd	Ferredoxin
GC	Gas Chromatography
gdcw	Gram dry cell weight of bacteria
HPLC	High-Pressure Liquid Chromatography
hup-	Uptake Hydrogenase Deficient
Lc	Liter-culture
NAD^+	Nicotinamide Adenine Dinucleotide
PBR	Photobioreactor
PHB	Poly-β-hydroxy butyrate
Pi	Inorganic Phosphate
PNSB	Purple Nonsulfur Bacteria
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin (flavodoxin) oxidoreductase

CHAPTER 1

INTRODUCTION

Fossil fuels are still the primary energy source of our times. However, these fuels are non-renewable and cause greenhouse gas emissions implicated in global warming. Biological hydrogen production is promising as a clean and sustainable route since it is renewable and bears the possibility of sunlight and waste utilization. Biological hydrogen production can be realized using various microorganisms, including cyanobacteria, green algae, facultatively anaerobic bacteria, and photosynthetic bacteria. Production of biohydrogen by photofermentation is realized by using photosynthetic purple sulfur bacteria (*Thiorhodoaceae*) (Maróti *et al.,* 2010) or purple nonsulfur bacteria (PNSB) (*Rhodobacteraceae*) (Basak *et al.,* 2007) under anaerobic conditions using different organic substrates with the presence of light. Biological photosynthetic hydrogen production using purple nonsulfur bacteria is a promising field as a clean hydrogen production route since it is renewable and bears the possibilities of fuel production from solar energy and utilization of simple sugars and organic acids such as acetate, butyrate, propionate, and lactate, and sugar-containing agricultural and industrial wastes.

1.1 Hydrogen, Characteristics, and Usage

Hydrogen, having atomic number 1, forms a diatomic H_2 molecule with another hydrogen atom by covalent bond formation. The hydrogen atom can also form covalent bonds with other elements such as carbon and forms CH_4, benzene, phenols, carbohydrates, cellulose, and lignin among the countless number of carbon complexes, with nitrogen, NH_3, N_2H_3, NH_2-N, and HNO_3, with oxygen, H_2O and H_2O_2, with sulfur, H_2S, H_2S_2, H_2S_3, and H_2S_5, with halogens HCl, HF, HBr and HI, and also with boron, different borohydrides, $NaBH_4$, $B_{10}H_{14}$, B_5H_5.

Therefore, hydrogen is considered the most abundant element in the world.
Hydrogen as a fuel has unique properties; when combusted, it produces H_2O, and the heat of combustion is 142 kJ/g. Hence is considered an environmentally clean energy carrier. One kg of hydrogen leads to 142 kJ of energy, equivalent to the combustion of 2.1 kg of natural gas and 2.8 kg of gasoline. Hence, it has high energy content per mass and is suitable for future transport fuel (Van Mierlo *et al.*, 2006). H_2 is an energy carrier like electricity. Besides being a clean energy carrier, it is also one of the important raw materials for the chemical and petroleum industry in gaseous and liquid forms.
Synthesis of ammonia, methanol, food industry hydrogenation of edible oils, and synthetic gas generation are among the industrial uses of hydrogen. It is also important in the steel, glass, ceramic and cement industries due to its high heat value. Hydrogen must be produced from other energy sources.

1.2 The H_2 Production Methods

Hydrogen production techniques include chemical, electrolytic, thermochemical, photolytic, and biological processes. For industrial-scale applications, hydrogen is produced from natural gas from petrochemical cracking (crude oil cracking) in two-stage processes: steam methane, propane reforming and water splitting, and thermochemical splitting. So H_2 is still produced from fossil fuels. Water electrolysis is usually performed using electrochemical energy obtained from fossil fuels. Therefore, hydrogen can not be considered as green energy and raw material.
Recently alternative biological-based methods to obtain H_2 from renewable sources, biomass, agricultural wastes, and household waste water have been developed and called biohydrogen. Algae and cyanobacteria can realize biological hydrogen production in the form of biophotolysis, thermophilic and mesophilic bacteria in the form of dark fermentation, and photosynthetic bacteria in the form of photofermentation, and by thermophilic and photosynthetic bacteria in the form of combined systems. Anaerobic bacteria convert organic compounds like glucose and

sucrose to hydrogen, carbon dioxide, and organic compounds like organic acids like acetate and butyrate during dark fermentation, which is a fermentative method for producing hydrogen. As opposed to this, photofermentation, or photo-fermentative hydrogen production, uses photosynthetic bacteria to convert organic acids into hydrogen and carbon dioxide when exposed to light.

1.2.1 Biological Hydrogen Production

Biological Hydrogen production technologies are based on microbial (metabolic) production of Hydrogen Biofuel using different microorganisms, including cyanobacteria, green algae, PNSB, purple bacteria, and facultative anaerobic bacteria.

Biological hydrogen production techniques can be studied in four basic parts: Biophotolysis and photofermentation, which can be described as light-driven biohydrogen production, Dark fermentation, and Microbial Electrolysis (Hallenbeck *et al.*, 2018).

1.2.1.1 Biophotolysis of Water

Under anaerobic conditions, algae and cyanobacteria break water molecules into hydrogen and oxygen through biophotolysis, which uses light as the driving force (Gaffron and Rubin, 1942). Equation (1.1) illustrates the entire biophotolysis reaction by algae, which is catalyzed by [FeFe]-hydrogenase.

Direct biophotolysis:

$$2\ H_2O\ (l) + \text{light} \rightarrow 2\ H_2\ (g) + O_2\ (g) \qquad (1.1)$$

Equation (1.2) illustrates the total biophotolysis reaction carried out by cyanobacteria and catalyzed by [NiFe]-hydrogenase.

Indirect biophotolysis:

$$6H_2O(g) + 6CO_2 \xrightarrow{hv} C_6H_{12}O_6(g) + 6O_2(g) \quad (1.2)$$

$$C_6H_{12}O_6(g) + 6H_2O(g) \xrightarrow{hv} 12H_2(g) + 6CO_2(g)$$

In addition to being inefficient, since biophotolysis requires an anaerobic environment, generated oxygen slows down the reaction by inhibiting the enzymes nitrogenase and hydrogenase (Ghirardi, *et al.*, 2007).

1.2.1.2 Dark Fermentation Method

The process of converting organic materials like glucose and sucrose to hydrogen, carbon dioxide, and lighter organic compounds like organic acids such as acetate and butyrate by anaerobic bacteria takes place during fermentative H_2 production, also known as dark fermentation. Equation (1.3) illustrates the hydrogenase-catalyzed dark fermentation reaction.

$$C_6H_{12}O_6\ (l) \rightarrow H_2\ (l) + CO_2\ (l) + \text{Organic acids} \quad (1.3)$$

When evaluating the benefits and drawbacks of dark fermentation, one advantage is that direct sunlight input is not necessary, making reactor design easier (Hallenbeck and Ghosh, 2009). Additionally, many wastewater types and agricultural wastes may be used in a dark fermentation process (Kapdan and Kargi, 2006). During the dark fermentation phase, valuable byproducts are produced, including acetic acid, butyric acid, and lactic acid. However, the dark fermentation's hydrogen yields are limited due to the organic molecules' partial breakdown (Hallenbeck and Ghosh, 2009).

1.2.1.3 Photofermentation Method

Photofermentation provides some advantages (Das *et al.,* 2001), including high theoretical conversion yields, utilization of broad light spectrum, and utilization of a wide variety of organic substrates, including organic substrates and agricultural wastes (Claassen *et al.,* 2010). Photofermentative hydrogen production has disadvantages, too, such as inadequate H_2 productivity and costly photobioreactors. In addition, photobioreactors that allow light penetration but not oxygen or nitrogen are needed (Fedorov *et al.,* 1998). Low light conversion efficiencies and high energy demands are also disadvantageous (Hallenbeck *et al.,* 2018).

Simple sugars and various organic substances, such as acetic acid, butyric acid, lactic acid, and waste products, could be used as substrates for growth and photo-fermentative hydrogen production by PNSB. Acetic acid has a key role in metabolism since it may be used directly as an energy source via the Krebs cycle or could be directed to poly hydroxy butyrate synthesis for storage (ÖzsoyDemiriz, 2019). The reaction of H_2 production by photofermentation using acetic acid, which may be obtained by dark fermentation as a substrate, is shown below in equation (1.4).

$$CH_3COOH + 2\ H_2O\ (l) + light \rightarrow 4\ H_2\ (g) + 2\ CO_2\ (g) \qquad (1.4)$$

1.3 Purple Non-Sulphur Bacteria (PNSB)

Biological hydrogen production can be realized using a variety of microorganisms, including green algae, cyanobacteria, purple sulfur bacteria, purple nonsulfur bacteria, green sulfur bacteria, green nonsulfur bacteria, and facultative anaerobic bacteria. PNSB has importance and advantage since they have a broad spectrum of growth modes such as photoautotrophic and chemoautotrophic modes, and they have metabolic flexibility such as photoheterotrophic, chemoheterotrophic, mixotrophic and fermentative metabolisms, hence they are meaningful choices for biological

hydrogen production (Elkahlout, 2011). Purple Nonsulfur Bacteria (PNSB), as α- and β-proteobacteria, belong to active nitrogen fixers, under light and anoxygenic conditions in nitrogen deficiency, produce H_2 since they consume simple organic acids such as acetic, propionic, and malic acids. They have been used in agricultural and wastewater treatments.

Purple nonsulfur bacteria (*Rhodobacteraceae*) (Basak and Das, 2007) can be used for biohydrogen production by anaerobic fermentation. PNSB can grow microaerobically or anaerobically, in the dark or under light, at a pH range of 6.0-9.0 and a temperature range of 25 C°-35 C°. They also produce PHB and carotenoids as byproducts. High conversion rates, the ability to use different organic substrates and even agricultural wastes, and benefiting from a wide range of the light spectrum are among the advantages of photofermentation (Claassen *et al.,* 2010). However, photofermentation leads to relatively lower hydrogen productivity and light conversion efficiencies. It requires high energy demands and costly photobioreactors that maintain light penetration but do not allow oxygen and nitrogen leakage (Fedorov *et al.,* 1998).

PNS bacteria can utilize various organic acids (such as acetate, lactate, and butyrate), industrial wastes, and agricultural wastes for growth and biological hydrogen production. Acetate can be consumed directly for energy purposes (TCA cycle) or storage purposes (Poly hydroxy butyrate (PHB) synthesis). Hence it is the key intermediate of the metabolism (ÖzsoyDemiriz *et al.,* 2019). The reaction of overall photo-fermentative H_2 production from acetate is found in the dark fermenter effluent. Various PNSB strains (Ozgur *et al.,* 2010, Hustede *et al.,* 1993) have been used for photo-fermentative hydrogen production from acetate, such as *R. capsulatus* (Özgür *et al.,* 2010, Boran *et al.,* 2010, Gebicki *et al.,* 2010); *Rp. palustris* (Carlozzi *et al.* 2012, Muzziotti *et al.* 2016); and *R. sphaeroides* (Basak *et al.* 2007, Sasikala *et al.* 1991, Kars *et al.* 2008, Eroğlu *et al.* 2008, Uyar *et al.* 2008, Akkose *et al.* 2009, Kars *et al.* 2009).

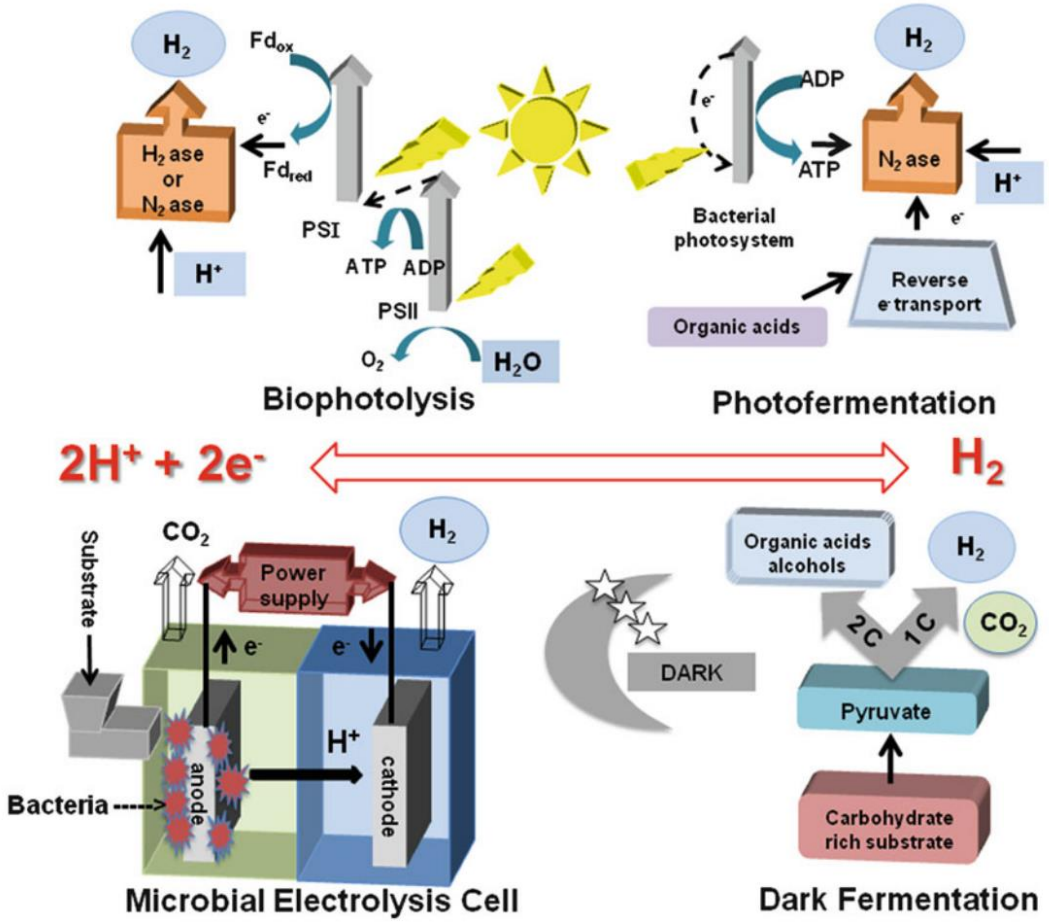

Figure 1.3.1 Biohydrogen Production Pathways: Biophotolysis, Photofermentation Dark fermentation and Microbial Electrolysis Cell (MEC) (Zannoni *et al.*, 2014).

1.4 Enzymes of Hydrogen Production in Purple Non-Sulphur Bacteria

The main enzymes of hydrogen metabolism are nitrogenase and hydrogenase. Under nitrogen-limited conditions, the nitrogenase enzyme catalyzes the formation of molecular hydrogen, whereas hydrogenase can produce or consume hydrogen.

1.4.1 Hydrogenase

Hydrogenases represent a set of crucial enzymes related to hydrogen metabolism and are present in most microorganisms capable of generating molecular hydrogen. In anoxygenic photosynthetic bacteria, the hydrogenase enzyme facilitates the reversible conversion of hydrogen, earning it the moniker "hydrogen-uptake hydrogenase." Hydrogenases in different organisms exhibit variations in their characteristics, such as molecular weight, the type of electron donors and acceptors they interact with, cellular arrangement, and other features. In *R. sphaeroides* and the majority of anoxygenic photosynthetic bacteria, hydrogenase primarily plays a role in hydrogen consumption. However, under specific conditions, including factors like Km values and bacterial preferences, they can also produce hydrogen (Van Haaster *et al.,* 2005). Various hydrogenase variants have been examined and categorized based on their metal ion composition, which encompasses [NiFe]-hydrogenases, [FeFe]-hydrogenases, and Fe-only Hydrogenase. [NiFe]-hydrogenases, which have been identified as the most abundant and extensively researched class of hydrogenases in bacteria (Vignais and Billoud, 2007), are a type of αβ heterodimer. They consist of a core structure featuring a large α-subunit that houses a bimetallic active site composed of [NiFe] and a small β-subunit that contains [Fe-S] clusters. The small β-subunit transfers electrons through its [Fe-S] clusters, while the large α-subunit catalyzes the reaction via its active site, which hosts the heterobinuclear [NiFe] metallocenter (Frey, 2002). Different subgroups of [NiFe] hydrogenases have been identified based on sequence analysis of their small and large subunits. These subgroups include membrane-bound uptake hydrogenases, hydrogen sensors, NADP-reducing hydrogenases, bidirectional NADP/NAD-reducing hydrogenases, and energy-converting membrane-associated hydrogen-evolving hydrogenases (Vignais and Billoud, 2007; Kovacs *et al.,* 2004; Vignais *et al.,* 2001). Membrane-bound respiratory hydrogenases perform the oxidation of hydrogen, followed by the reduction of quinone molecules. During anaerobic respiration, various electron acceptors are utilized, such as NO_3^-, SO_4^{-2}, fumarate, or

carbon dioxide. In aerobic respiration, oxygen serves as the final electron acceptor. The energy generated is then harnessed in the form of a proton motive force after the electrons are transferred to the final acceptors. In this manner, uptake hydrogenases play a role in maintaining the cell's energy balance. The primary factors influencing the activity of uptake hydrogenase are oxygen levels, hydrogen availability, and the presence of essential metal ions like iron and nickel. Transcriptional analyses have been conducted to investigate how oxygen, hydrogen, and nickel affect the expression levels of [NiFe]-hydrogenases (Tamagnini *et al.*, 2002; Boison *et al.*, 2000). These studies have shown that increased levels of both hydrogen and nickel lead to higher hydrogen-uptake hydrogenase activity. Additionally, reduced oxygen levels impact uptake hydrogenase activity, and the presence of hydrogen triggers the expression of certain hydrogenases through hydrogen-sensing regulatory hydrogenase and a two-component system. The specific effects of hydrogen on hydrogenase expression were further examined in detail in R. capsulatus (Dischert *et al.*, 1999). Uptake hydrogenase is a membrane-bound enzyme that consumes hydrogen (H_2). In the literature, there are studies related to the improvement of photo-fermentative hydrogen production by using some strategies, such as the elimination of uptake hydrogenase (*R. capsulatus* hup- (YO3) (Ozturk *et al.,* 2006), and *R. sphaeroides* hup- (Kars *et al.,* 2008)). Compared with the wild types, those hup-mutants showed improvement in H_2 production. Hox hydrogenases, on the other hand, which are metalloenzymes, reversibly oxidize hydrogen or reduce protons and require the presence of electron donors with low ionization potential to catalyze hydrogen evolution (Hallenbeck *et al.,* 2019, Özgür *et al.,* 2010). Another type of hydrogenase, which is membrane-bound, found in PNSB, namely uptake hydrogenases, consume H_2 and, as shown in the literature, elimination of the uptake hydrogenase leads to improvement in photo-fermentative hydrogen production (Öztürk *et al.*, 2006, Kars *et al.*, 2008). The general equation for hydrogenase activity is shown below in equation (1.6).

$$H_2 \quad \leftrightarrow \quad 2\,H^+ + 2\,e^- \qquad (1.6)$$

1.4.2 Nitrogenase

The formation of molecular H_2 is catalyzed by nitrogenase under N_2-diminished conditions. That is an energy-requiring process (ATP) produced by photophosphorylation, the TCA cycle, and the photosynthetic membrane apparatus complements the hydrogen metabolism of the PNSB (Vignais *et al.,* 1985, Sasikala *et al.,* 1990, Koku *et al.,* 2002). Also, oxygen inhibits its enzyme activity. The equation for nitrogenase activity in the presence of N_2 is shown below in equation (1.7), and the equation for nitrogenase activity under N_2 limitation and anaerobic environment is shown below in equation (1.8).

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ Pi \quad (1.7)$$

$$8\ H + 8\ e^- + 16\ ATP \rightarrow 4\ H_2 + 16\ ADP + 16\ Pi \quad (1.8)$$

PNSB nitrogenases may vary with the species, such as Mo-, V-, or Fe-dependent nitrogenases. Expressing particular forms of each nitrogenase is advantageous for hydrogen production. The better hydrogen producer strains of PNSB species are important. *Rp. palustris* (CGA 009) remains the only example of a photosynthetic membrane that encodes all three nitrogenase proteins: Mo only, V only, and Fe only, which grow slowly but have higher specific productivity. However, Hox hydrogenase enzymes reversibly catalyze the oxidation of hydrogen or reduction of the protons for hydrogen production. These enzymes are also metalloenzymes, catalyzing the formation of hydrogen molecules when electron donors with low ionization potential are present (Hallenbeck *et al.,* 2018, Ozgur *et al.,* 2010).

1.5 Co-cultures of Purple Non-Sulphur Bacteria

Limitations of biological hydrogen production are low productivity, low efficiency, high photobioreactor costs, low feasibility, low light conversion efficiencies, and high energy demand by nitrogenase. To realize hydrogen production more efficiently, different strategies may be followed, including studying productivity with different feedstocks, strain improvement with genetic engineering, immobilizing the consortium (Elkahlout *et al.,* 2018, Sagir *et al.,* 2018), innovations regarding the reactors (Androga *et al.,* 2017, Kayahan *et al.,* 2017), further expressional and metabolical studies regarding H_2 (Gürgan *et al.,* 2015, Erkal *et al.,* 2019), and mixed (Sasikala *et al.,* 1994), double (Kondo *et al.,* 2002, Okubo *et al.,* 2007) and triple co-cultivation of PNSB. *R. capsulatus* (Özgür *et al.,* 2010, Boran *et al.,* 2010, Gebicki *et al.,* 2010); *Rp. palustris* (Carlozzi, 2012, Muzziotti *et al.,* 2016); and *R. sphaeroides* (Sasikala *et al.,* 1991, Basak *et al.,* 2014, Kars *et al.,* 2008, Eroğlu *et al.,* 2008, Uyar *et al.,* 2008, Akkose *et al.,* 2009, Kars *et al.,* 2009) are among the PNSB strains encountered in the literature concerning photo-fermentative hydrogen production from acetate. When *Halobacterium salinarum* packed cells were used together with *R. sphaeroides* O.U.001, hydrogen production was increased 4 to 6-fold (Zabut *et al.,* 2006). The mutation by heterologous expression of chlorophyll a synthase in *R. sphaeroides* has led to a 13.6 and 22.6% increase in hydrogen yield and productivity, respectively (Ipekloğlu *et al.,* 2016). NH_4, acetic acid, and O_2 concentrations have a role in the regulation of nitrogenase, therefore influencing hydrogen yield and productivity of hydrogen production by *R. sphaeroides* (Akkose *et al.,* 2009) and the use of ammonium chloride or glutamic acid as the N source influences photoheterotrophic metabolism of *R. capsulatus* (Erkal *et al.,* 2019). In the literature, studies regarding the biological hydrogen production of PNSB co-cultivated with fermentative bacteria, including *C. butyricum* and *Rp. palustris* also exist (Hitit *et al.,* 2016). The influence of such co-cultures on pH has been studied (Zagrodnik *et al.,* 2016). Hydrogen production by co-cultivated *C. acidisoli* and *R. sphaeroides* on sucrose has also been experimented (Sun *et al.,* 2010). Also, the co-

cultivation of *Rp. palustris* and cyanobacteria *Anabaena sp.* in favor of hydrogen production has been studied (Wu *et al.,* 2011). In addition to the studies utilizing co-cultures, there have also been efforts investigating the hydrogen production potentials of mixed cultures (Tawfik *et al.,* 2014). One study used mixed cultures in the form of anaerobic granular inocula on dairy wastewater substrates (Koroglu *et al.,* 2019). Mixed purple nonsulfur bacteria cultures have been implied to increase hydrogen yields on agricultural wastes (Argun *et al.,* 2009). Co-cultivation of PNSB has also been studied on whey protein and glucose as a substrate to realize H_2 production (Machado *et al.,* 2018).

1.6 Improvement of Hydrogen Production

Many strategies for improving hydrogen production by PNSB have been studied and shown in the literature. Immobilization of the PNSB may lead to more efficient hydrogen production (Elkahlout *et al.,* 2008, Sagir *et al.,* 2018), new reactor design (Androga *et al.,* 2017, Kayahan *et al.,* 2017), a better understanding of gene expression and metabolism of hydrogen (Gürgan *et al.,* 2015, Erkal *et al.,* 2019, Gürgan *et al.,* 2018), and co-cultivation of different PNSB such as mixed culture (Sasikala *et al.,* 1994), two different species of phototropic PNSB (Kondo *et al.,* 2002, Okubo *et al.,* 2007), in a co-culture system with acetate utilization (Hitit *et al.,* 2017) and with molasses utilization (Sagir *et al.,* 2017). Biohydrogen production by co-cultures of fermentative and PNSB such as *Clostridium butyricum* and *Rp. palustris* have also been studied (Hitit *et al.,* 2017). The effect of pH on co-cultures of *Clostridium acetobutyricum* and *R. sphaeroides* has been investigated (Zagrodnik *et al.,* 2006). Another study focused on optimizing H_2 production by a co-culture of *Clostridium acidisoli* and *Rhodobacter sphaeroides* with sucrose as substrate (Sun *et al.,* 2009). Co-cultures of *Rp. palustris* and cyanobacteria *Anabaena sp.* have also been utilized for biohydrogen production (Wu *et al.,* 2011). In addition to the studies investigating the effect of co-cultures in hydrogen production, there are also studies investigating the effect of mixed cultures in hydrogen production (Tawfik *et al.,*

2014). Also, one study utilized anaerobic granular inoculums containing mixed bacteria cultures for hydrogen production from dairy wastewater (Koroglu *et al.*, 2019). PNSB mixed cultures have been proposed for obtaining higher H_2 yields on agricultural wastes (Argun *et al.,* 2009). Photofermentative hydrogen production on milk whey protein and glucose by co-cultures of *Rp. palustris* and *R. capsulatus* have been investigated (Machado *et al.,* 2018).

1.7 Immobilization

Immobilization refers to the localization or confinement of cells to a certain area of space to maintain a specified catalytic activity (Karel *et al.,* 1985). Immobilization confers some advantages to photofermentation process feasibility and efficiency (Zhang *et al.,* 2010, Tsygankov *et al.,* 2001, Fibler *et al.,* 1995). Costs associated with cell recovery and recycling are reduced since high cell concentrations are maintained, and cells can be reused without re-cultivation. Continuous cultures decrease cell wash-out issues, and increased cell concentrations, in combination with high flow rates, can produce high volumetric productivities. Additionally, it is possible to create favorable microenvironments, enhance genetic stability, and prevent shear damage brought on by reactor mixing or aeration. In addition, if the bacteria are immobilized inside an entrapment medium such as agar, contamination of bacteria and medium toxicity is prevented or mitigated by a physical barrier. Therefore this type of hydrogen production is also suitable for waste utilization. Immobilization also confers the benefits of easier pH and temperature control and easily filtering out inhibitors from the medium. The primary drawbacks of the immobilization technique, on the other hand, are as follows: Product may need to be collected from the cells, problems may occur owing to diffusional restrictions, it may be challenging to regulate the microenvironment due to heterogeneity, and growth of the bacteria and gas evolution may disturb the immobilized matrix, among other factors (Elkahlout, 2011). Agar, reverse micelle microreactors with hydrophilic centers and hydrophobic groups spreading out, and immobilization on glass surfaces

are some experimental techniques (Tsygankov *et al.,* 1993, Singh and Misra, 2009, Elkahlout, 2011). Reverse micelle reactor use has resulted in a 50-fold increase in hydrogen production rate by *R. palustris* (Singh and Misra, 2009). For *R. capsulatus* DSM 1710 and YO3, agar immobilized systems with concentrations of 3 and 4% have shown the best results. There have been reports of diffusion restrictions for products and nutrients at higher agar concentrations. Since 4 mM glutamate produced better outcomes than 2 mM glutamate and 60 mM acetate produced the best yields, a greater nitrogen supply was also necessary. For *R. capsulatus* YO3 and DSM 1710, respectively, the maximum substrate yields were reported to be 3.4, 3.4, 2.4, and 2.3, 3.1, and 3 mmol H2 / mmol acetate. Additionally, co-immobilization of agar immobilized systems with H. salinarium led to production increases of 1.14–1.41 times (Elhahlout, 2011).

Numerous experiments have shown that PNS bacteria can produce hydrogen when co-cultured and grown alone in an immobilized environment (Basak *et al.,* 2014, Tiang *et al.,* 2020).

A summary of Recent Studies on Biological Hydrogen Production by Immobilized Photosynthetic Bacteria by Sagir and Alipour (2021) is given below in Table 1.7.1.

Table 1.7.1 Recent studies on biological hydrogen production by immobilized photosynthetic bacteria (Sagir and Alipour, 2021)

Bacteria	Immobilized material	Substrate	Substrate concentration (g/L)	Reactor type	Reactor volume (mL)	Light source	Light intensity	Process type	Hydrogen production rate (mLH2/L/h)	Hydrogen yield (mol H_2/mol substrate)	Reference
Rhodobacter capsulatus DSM 1710	Agar	Acetic acid	3.6	Roux bottle	200	Tungsten	4000 lux	Fed-batch	25.7a	2.92 mol H_2/mol acetate	Elkahlout *et al.*, 2016
Rhodobacter capsulatus YO3	Agar	Acetic acid	3.6	Roux bottle	200	Tungsten	4000 lux	Fed-batch	48.9a	3.08 mol H_2/mol acetate	Elkahlout *et al.*, 2016
Rhodopseudomonas faecalis RLD-53	ACF	Acetic acid	4.1	Glass vessel	100	Incandescent	150 W/m2	Batch	32.85	3.08 mol H_2/mol acetate	Xie *et al.*, 2012
Rhodobacter capsulatus DSM 1710	Agar	Acetic acid	3.6	Plexiglas panel	1400	Tungsten	200 W/m2	Fed-batch	18.0a	2.51 mol H_2/mol acetate	Elkahlout *et al.*, 2018
Rhodobacter capsulatus YO3	Agar	Acetic acid	3.6	Plexiglas panel	1400	Tungsten	200 W/m2	Fed-batch	31.2a	3.53 mol H_2/mol acetate	Elkahlout *et al.*, 2018
Rhodobacter sphaeroides IL106	Agar	Acetic acid	1.25	Roux bottle	200	Halogen	0.19 mEinstein/m2/s	Batch	18	3.03 mol H_2/mol acetate	Asada *et al.*, 2008
Rhodopseudomonas faecalis RLD-53	Agar	Glucose	9	Serum bottle	100	Incandescent	2000 lux	Batch	9.1	6.32 mol H_2/mol glucose	Liu *et al.*, 2009
PSB Consortium	Alginate	Synthetic wastewater	5.5b	Conical flask	250	Incandescent	8000 lux	Batch	8.74	N.A.	Zhang *et al.*, 2017
Rhodobacter sphaeroides O.U.001	Porous glass	Malic acid	2	Glass PBR	235	Vitalux	102 W/m2	Continuous HRT, 22 h	12.7	2–6 mol H_2/mol malic acid	Zagrodnik *et al.*, 2015
Rhodopseudomonas palustris CQK 01	Glass bead	Glucose	4	PMMA vessel	1200	LED	5000 Lux	Batch	38.9	0.2 mol H_2/mol glucose	Tian *et al.*, 2010
Rhodobacter sphaeroides O.U.001	Porous glass	Malic acid	2	Flat plate	200	Vitalux	64 W/m2	Semi-continuous	59	4.2 mol H_2/mol malic acid	Zagrodnik *et al.*, 2013
Rhodobacter capsulatus YO3	Agar	Sucrose	1.7	Plexiglas panel	1400	Tungsten	200 W/m2	Fed-batch	17.8a	19 mol H_2/mol sucrose	Sagir *et al.*, 2017
Rhodobacter capsulatus YO3	Agar	Beet molasses	1.7	Plexiglas panel	1400	Tungsten	200 W/m2	Fed-batch	18.9a	12.2 mol H_2/mol sucrose	Sagir *et al.*, 2018
Rhodopseudomonas palustris CQK 01	PVA, alginate and carrageenan	Glucose	4	Flat panel PMMA	800	Tungsten	3000 lux	Continuous HRT, 11.4 h	62a	0.74 mol H_2/mol glucose	Wang Sagir *et al.*, 2013

This study examines the synergistic effect of employing co-cultures of PNS bacteria as well as the possible benefits of using an immobilization system. It focuses on hydrogen production using co-cultures of PNS bacteria in an immobilized setting.

1.8 Photobioreactors

The model of the reactor is a crucial factor in determining hydrogen productivity and production. Photobioreactors must keep products, substrates, and cells contained and free from contamination (Tredici, 2004). High light availability and an ideal area-to-volume ratio are crucial in reactor design. As a result, light energy may better pass-through cells, be used to its fullest extent per culture volume, and be maintained with improved light distribution across the reactor volume (Akkerman *et al.,* 2002). Although they would be vulnerable to shocks, thin surfaces would have excellent light penetration.

On the other hand, thick surfaces would be more robust but more costly and less light-permeable. Better light dispersion would come from high surface-to-volume ratios. The illuminated area to cultured medium ratio would remain low, and the solar would not benefit entirely since part of the penetrating light would escape the photobioreactor. However, large surface areas to volume ratios demand smaller reactor sizes. On the other hand, relatively low surface-to-volume ratios provide just a narrow region of light penetration, leaving a significant portion of the culture dark. High cell densities also lead to restricted light penetration zones, light inhibition, light saturation, and light restriction, all of which may coexist in the same reactor configuration. Such a situation can be resolved via bidirectional mixing, which involves transferring cells between various zones (Ogbonna and Tanaka, 2001).

Several different types of reactors, including panel photobioreactors and tubular photobioreactors, are used to produce hydrogen photo-fermentatively. Panel bioreactors are rectangular, transparent containers with a depth of 1 to 5 cm (Akkerman *et al.,* 2002). Although they are often positioned vertically, they can also be tiled at the best angles to get the most sunlight (Tredici and Zitelli, 1997,

Richmond *et al.,* 1999). Additionally, flat reactors may be stacked closely together to create an effect known as lamination that dilutes solar radiation five times, increasing biomass conversion efficiency (Carlozzi, 2000, Richmond and Cheng, 2001). The absence of stirring devices in panel photobioreactors is their main flaw. However, mixing may be done with bubbling gas (Akkerman *et al.,* 2002).

On the other hand, transparent tubes with diameters ranging from 3 to 6 cm constitute tubular photobioreactors. The surface area to volume ratio is larger in tubular photobioreactors. Pumps are used to keep the cultures flowing. According to Akkermann *et al.,* (2002), tubes can be installed in a variety of ways, including: horizontally as straight tubes with u-bends; vertically by bending as a cylinder or cone coils; vertically as fence-like structures with u-bends or connecting manifolds; and horizontally as parallel tubes connected by manifolds.

Bacteria that produce hydrogen has been tested in continuous-operating tube and panel photobioreactors (Sarı 2007, Uyar 2008, Androga 2009, Boran 2011). Sarı (2007) reported a hydrogen generation rate of 0.009LH_2/Lc.h in helical tubular bioreactors with *R. sphaeroides* O.U. 001. On the other hand, Uyar (2008) observed hydrogen generation rates of 0.52 and 0.27 mg/Lc.h using panel bioreactors with *R. capsulatus* (hup-) YO3 and DSM 1710, respectively. Using panel bioreactors with *R. capsulatus* (hup-) YO3, Androga (2009) produced 0.4 mmol H_2 per Lc.h. *R. capsulatus* (hup-) YO3 and DSM 1710 were used in tubular bioreactors by Boran (2011) to produce 0.2 and 0.3 mmol H_2/m^3.h, respectively.

Previously, in METU Hydrogen Research Laboratory, hydrogen production with panel reactors, continuous tubular reactors, u-bends, helical bioreactors, and cylindrical reactors was experimented with, as shown in Figure 1.8.1.

Figure 1.8.1 Panel reactors, continuous tubular reactors, and tubular reactors with u-bends as some of the previously experimented reactor types in the METU Hydrogen Research Laboratory (Androga *et al.*, 2012, Kayahan *et al.*, 2016)

In this study, an alternative bioreactor type was designed, which is made of glass, cylindrical in shape, and internally covered with bacteria immobilized with agar, to optimize sunlight utilization with varying incidence angles throughout the day and the year and to prevent cell wash-out and loss, and to maintain re-use of bacterial cultures.

1.9 Aim

This study focused on hydrogen production by agar immobilized different single, double, and triple co-cultures of PNS Bacteria, namely, *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in a novel photobioreactor to improve stability for bacterial activity and hydrogen productivity provided by immobilization in indoor and outdoor conditions. The study investigates both the potential synergistic effect of using co-cultures of PNSB and aims to improve hydrogen yield per feedstock, decrease the necessity of resetting the production system by cleaning and re-inoculation with a fresh bacterium due to the prevalence of other microorganisms by time, decrease maintenance costs in contrast to non-immobilized systems. The growth and hydrogen production performances of those combinations were investigated using acetate and glutamate as the carbon and nitrogen sources, respectively. The agar immobilized different bacterial systems were tested in three different photobioreactors: i) 1.4 L Plane PBR ii) 300 mL PBR and iii) Novel cylindrical glass PBR. The effects of light intensity and acetate concentration were tested in 150 mL reactors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains

In this study, three different PNSBs were used: *R. capsulatus* hup- (YO3), a mutant strain lacking the uptake hydrogenase, was obtained by genetic modification by Dr. Yavuz Öztürk from *R. capsulatus* MT 1131 (Öztürk *et al.,* 2006), *Rp. palustris* (DSMZ 127), *R. sphaeroides* O.U.001 (DSMZ 5864).

2.2 Culture Media

Bacteria stored at -80 °C were spread on mineral-peptone-yeast extract (MPYE) agar plates to check against contamination. The 0.5 mL of bacterial stocks was first added to 1 mL of growth media and was exposed to 2000 lux light intensity at 30 °C. Following growth until an OD_{660nm} value of 2.0, cultures were transferred several times by 10% inoculation to fresh media to ensure adequate activity of the bacteria while successively increasing the culture volume to 15 mL and 50 mL. A standard BP growth medium (Biebl *et al.,* 1981) containing 20 mM acetate and 10 mM glutamate was prepared. The hydrogen production medium had standard BP media with 40 mM acetate and 2 mM glutamate. The composition of BP media was given in Appendix A.1. The composition of vitamin solution was given in Appendix A.2. The composition of trace elements solution was given in Appendix A.3.

2.3 Experimental Setup for Hydrogen Production

Different PNSB strains were grown, co-cultured, and immobilized simultaneously by mixing different combinations of the strains with agar, pouring them inside the lateral surfaces of 150 mL cylindrical bioreactors, and letting them solidify.

2.3.1 Growth of Bacterial Strains

Three different PNSB strains were used for this study. Namely: *R. capsulatus* hup- (YO3), a mutant strain lacking the uptake hydrogenase, *Rp. palustris* (DSMZ 127), *R. sphaeroides* O.U.001 (DSMZ 5864). *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunsweig Germany). *R. capsulatus* hup- (YO3) was obtained by genetic modification from *R. capsulatus* 1131 during our previous studies (Öztürk *et al.*, 2006). PNSB strains used were stocked at -80 °C. Firstly spreading on mineral-peptone-yeast extract (MPYE) agar plates were performed. Spread bacteria were checked against contaminations on agar plates. Following the contamination check, 0.5 mL of stocked bacterial cultures were mixed with 1.0 mL of 20/10 mM Acetate/Glutamate containing BP growth media and cultivated under 2000 lux light intensity at 30 °C. When the cultures had grown until obtaining an OD660 value of 2.0, they were transferred into 15 mL vials and supplemented with 13.5 mL of 20/10 mM Acetate/Glutamate containing BP growth media to establish 10 % inoculation. Following growth until an OD660 value of 2.0, 5.0 mL of the cultures were transferred into 50 mL vials. Again, following growth until an OD660 value of 2.0, cultures were transferred several times by 10% inoculation to fresh media to ensure adequate activity of the bacteria. An anaerobic environment was established by purging the air with argon, and the illumination of 2000 lux was provided using tungsten lamps. The temperature was maintained at 30 °C with a cooling incubator.

2.3.2 Immobilization of Bacteria

Single cultures and double and triple co-cultures of the strains were immobilized. Experiments were conducted in 1.4 L panel reactors, 300 mL panel reactors and 150 mL round reactors on 40 mM/2 mM Acetate/Glutamate medium, indoors and outdoors, and 60 mM/2 mM Acetate/Glutamate medium, indoors only, under anaerobic conditions. To prepare the production systems, *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) cultures grown on a defined growth medium until OD was 2.00 at 660 nm were concentrated by centrifugation at 9000 rpm and 13131 rcf for 20 minutes. Bacterial pellets were collected and resuspended into the water to obtain a cell concentration of 10.0 mg DCW/mL and maintained at 30 °C. 8% agar was prepared by mixing 80 g agar per liter of liquid volume and kept at 50 °C. The agar-bacteria matrix was prepared by swiftly mixing a hot agar mixture with an equal volume of bacteria-water mixture to prevent bacteria from stress or death and maintain bacterial health. Therefore, 4 % final agar concentration was achieved.

2.3.3 Photobioreactors for Immobilized System

For this study, firstly a 1.4 L plexiglass panel reactor type, which was previously designed and manufactured in our laboratory was utilized (Elkahlout *et al.*, 2018, Sagir *et al.,* 2018). 200 mL of bacteria-agar mixture was poured into rectangular compartment frames of these reactors, which were designed to bear the immobilized culture according to previous study mentioned. The bacteria-agar mixture contained 5 mg/mL dry cell weight gel culture per volume of agar. The single cultures, *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) included 5.0 mg DCW/mL gel culture from either of the strains, by containing 200 mL of agar-bacteria mixture for each one, the mixed co-culture of *Rp. palustris* (DSMZ 127) + *R. sphaeroides* O.U.001 (DSMZ 5864) included 2.5 mg DCW/mL gel culture of both strains, by containing 100 mL of agar-bacteria mixture for each one, mixed together

before pouring to the frames and solidifying, and the bi-layer co-culture of *Rp. palustris* (DSMZ 127) + *R. sphaeroides* O.U.001 (DSMZ 5864) included 2.5 mg DCW/mL gel culture of both strains, by containing 100 mL of agar-bacteria mixture for each one, by temporarily filling half of the compartment frames by rubber sheets, pouring the agar-bacteria mixture for one strain, then after the poured agar-bacteria mixture solidifies, removing the temporary rubber sheets from the compartment frames, and pouring the other agar-bacteria mixture, then waiting for solidification again. Reactors were filled with 1 L of 40 mM/2 mM Acetate/Glutamate medium, and hydrogen production experiments were performed outdoors. The initial pH was set to 6.3. The sunlight illuminated outdoor experiments. A Heto OBN 28 water bath with a Heto HWT 100 thermostatic heater and a Hetofrig CB11e cooling water bath were used to keep the temperature of the reactors at 30ºC. Temperatures of the reactors were controlled daily with external thermometers. All experimental groups were run as triplicates.

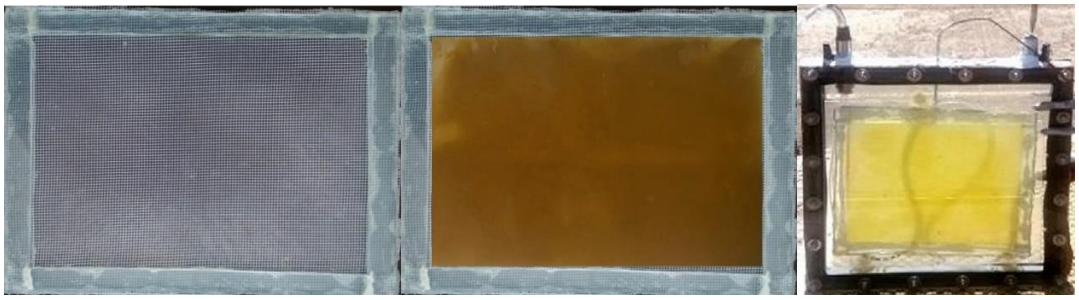

Figure 2.3.1 Rectangular compartment frame for the 1.4 L panel reactors, empty, filled with bacteria-agar mixture, and 1.4 L panel reactor, during experimentation.

Next, 300 mL plexiglass cell culture flasks were used as reactors. 50 mL of agar-bacteria mixture were poured into these flasks, and let to solidify. The bacteria-agar mixture contained 5 mg/mL dry cell weight gel culture per volume of agar. The single cultures, *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) included 5.0 mg DCW/mL gel culture from either of the strains, by containing 50 mL of agar-bacteria mixture for each one, the mixed co-culture of *Rp. palustris*

(DSMZ 127) + *R. sphaeroides* O.U.001 (DSMZ 5864) included 2.5 mg DCW/mL gel culture of both strains, by containing 25 mL of agar-bacteria mixture for each one, mixed together before pouring to the frames and solidifying, and the bi-layer co-culture of *Rp. palustris* (DSMZ 127) + *R. sphaeroides* O.U.001 (DSMZ 5864) included 2.5 mg DCW/mL gel culture of both strains, by containing 25 mL of agar-bacteria mixture for each one, by pouring the agar-bacteria mixture for one strain, then after the poured agar-bacteria mixture solidifies, pouring the other agar-bacteria mixture, then waiting for solidification again. Reactors were filled with 200 mL of 40 mM/2 mM Acetate/Glutamate medium, and hydrogen production experiments were performed indoors and outdoors. The initial pH was set to 6.3. Indoor experiments were conducted under 2500 lux illumination by a tungsten lamp. The sunlight illuminated outdoor experiments. An outdoor transparent water bath was designed for outdoor cooling/heating. Hence the temperature was maintained at the desired value, regardless of the outdoor temperature variations. The temperature for the indoor and outdoor experiments was set to 30ºC. For indoor experiments, a Nüve ES 250 cooling incubator was used. For outdoor experiments, a Heto OBN 28 water bath with a Heto HWT 100 thermostatic heater and a Hetofrig CB11e cooling water bath were used to keep the temperature of the transparent water baths at 30ºC. Temperature of the water bath was controlled daily with external thermometers. All experimental groups were run as triplicates.

Figure 2.3.2 300 mL panel plexiglass reactors inside the water bath during the outdoor experimentation.

For the third part of this study, the inside surface of a novel glass cylindrical reactor was covered with the immobilized agar-bacteria matrix to optimize light utilization throughout different times of the day and the year. To prepare this novel reactor setup, agar-bacteria matrix was poured into 150 ml glass cylindrical reactors of 66 mm diameter and 102 mm height and solidified on the inner lateral surface of the reactor by rotating the reactor in a tilted orientation (Figure 2.3.3). Laterally covering the inner surface of a cylindrical reactor is a new technique that enables optimal light utilization at every angle of incidence throughout the day, similar to suspension cultures. The final concentration of bacteria was 5 mg/mL dry cell weight gel culture per volume of agar. The thickness of the bacteria-agar layers was measured as 2 mm. Hence allowed light penetration throughout the reactors. A total of 24 gas transfer slits were cut on each agar layer in the shape of a plus (+) sign, with 1 cm width and 1 cm height, horizontally 8 per layer diameter, and 3 per layer height. The single cultures, *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) included 5.0 mg DCW/mL gel culture from either of the three strains, 30 ml each, the double co-cultures *R. capsulatus* hup- (YO3) + *Rp. palustris* (DSMZ 127), *R. capsulatus* hup- (YO3) + *R. sphaeroides* O.U.001 (DSMZ 5864), and *Rp. palustris* (DSMZ 127) + *R. sphaeroides* O.U.001 (DSMZ 5864) included 2.5 mg DCW/mL gel culture each from two of three strains, 15 ml from each, and the triple co-cultures *R. capsulatus* hup- (YO3) + *Rp. palustris* (DSMZ 127) + *R. sphaeroides* O.U.001 (DSMZ 5864) included 3.33 mg DCW/mL gel culture each from all three strains. Reactors were filled with 120 mL of 40 mM/2 mM or 60 mM/2 mM Acetate/Glutamate medium, and hydrogen production experiments were performed indoors and outdoors. The initial pH was set to 6.3. Indoor experiments were conducted under 2500 lux or 4000 lux illuminations by a tungsten lamp. The sunlight illuminated outdoor experiments. The outdoor transparent water bath designed previously for outdoor cooling/heating was used. The temperature for the indoor and outdoor experiments was set to 30ºC. For indoor experiments, a Nüve ES 250 cooling incubator was used. For outdoor experiments, a Heto OBN 28 water bath with a Heto HWT 100 thermostatic heater and a Hetofrig

CB11e cooling water bath were used to keep the temperature of the transparent water baths at 30ºC. Temperature of the water bath was controlled daily with external thermometers. All experimental groups were run as triplicates.

For the all parts of these experiments, experiments have continued as long as gas production has continued. Afterwards, reactors were emptied, refilled, and incubated with 20/10 Acetate/Glutamate growth medium for 48 hours. Then they were emptied again and refilled with hydrogen production medium to run another cycle of hydrogen production experimentation. The 60/2 and 40/2 Acetate/Glutamate hydrogen production media and the 20/10 Acetate/Glutamate growth media contained iron citrate, vitamins, and trace elements. The total gas produced was collected using 60 ml syringes. The gaskets of the syringes were lubricated by sterilized glycerol. Gas composition and pH measurements were performed every 24^{th} hour.

Figure 2.3.3 150 mL glass reactors covered internally with agar-bacteria matrix of 2 mm thickness.

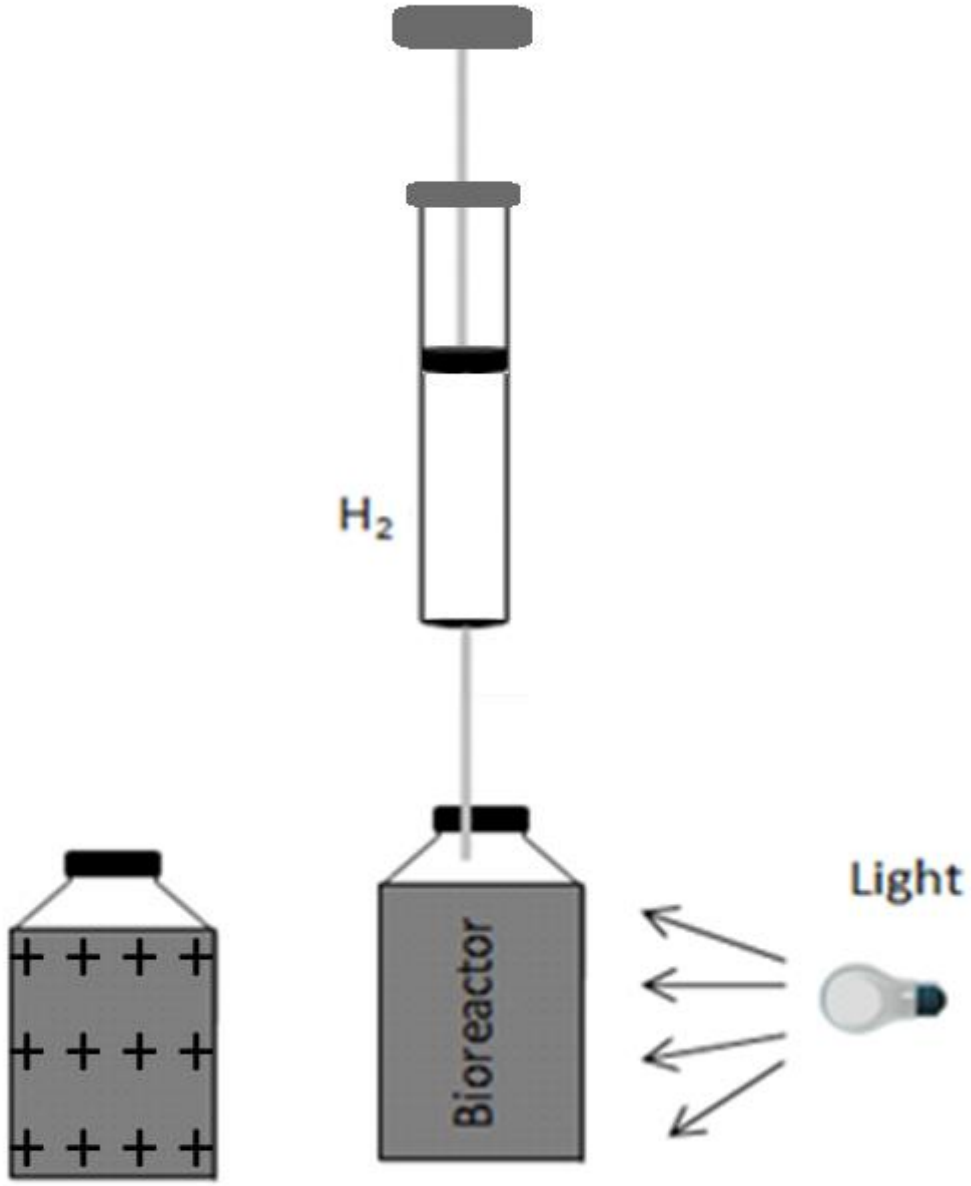

Figure 2.3.4 Schematic representation of the experimental setup depicting the gas transfer slits cut, and the bioreactor, gas collection, and illumination.

2.4 Sample Collection and Analysis

Analyses performed include cell concentration, pH, gas composition, and organic acid, as described below.

2.4.1 Cell Concentration Analysis

Optical densities (OD) were measured at 660 nm by a spectrophotometer (Shimadzu UV 1800). Dry cell weights were calculated by OD_{660nm} to dry cell weight conversion factors reported, such as 0.4656 $(g.L^{-1})/OD_{660nm}$ for *R. capsulatus* hup- (YO3) (Öztürk *et al.*, 2006), 0.5427 $(g.L^{-1})/OD_{660nm}$ for *R. capsulatus* (DSMZ 1710) (Uyar *et al.*, 2008), 0.2605 $(g.L^{-1})/OD_{660nm}$ for *Rp. palustris* (DSMZ 127) (Sagir et al., 2017), and 0.5781 $(g.L^{-1})/OD_{660nm}$ for *R. sphaeroides* O.U.001 (DSMZ 5864) (Eroğlu *et al.*, 2008). The mean of the OD_{660nm} to dry cell weight conversion factors was used to determine the total biomass of co-cultures.

2.4.2 pH Analysis

pH measurements were carried out by a Mettler Toledo 3311 pH meter.

2.4.3 Gas Composition Analysis

H_2, CO_2, and air composition analysis were performed using an Agilent Technologies 6890N GC device utilizing a SupelcoCarboxen 1010 gas column.

2.4.4 Organic Acid Analysis

Acetic acid, lactic acid, propionic acid, formic acid and isobutyric acid levels were measured during process by a Shimadzu LC-20A Prominence HPLC system using an Alltech IOA-1000 (300 mm x 7.8 mm) column and a UV-VIS detector (Shimadzu SPD-20AV) at 210 nm wavelength.

2.5 Data Analysis and Calculations

For the evaluation of hydrogen production results, hydrogen yield, substrate conversion efficiency and hydrogen productivities were calculated. The formulas were given in Appendix D, E and F.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Growth of PNSB Bacteria

In order to obtain the PNSB bacteria to be used in immobilization studies, bacteria from -80°C were grown in 20/10 mM Acetate/Glutamate medium. Biomass accumulation, pH variation and hydrogen production were monitored. Bacteria were repeatedly grown and transferred to new culture media until desirable growth and pH variation profile was obtained to ensure healthy metabolic activity. When the profiles were observed as expected, as given in Figure 3.1.1, cultures further were grown in volume and the experimentations began.

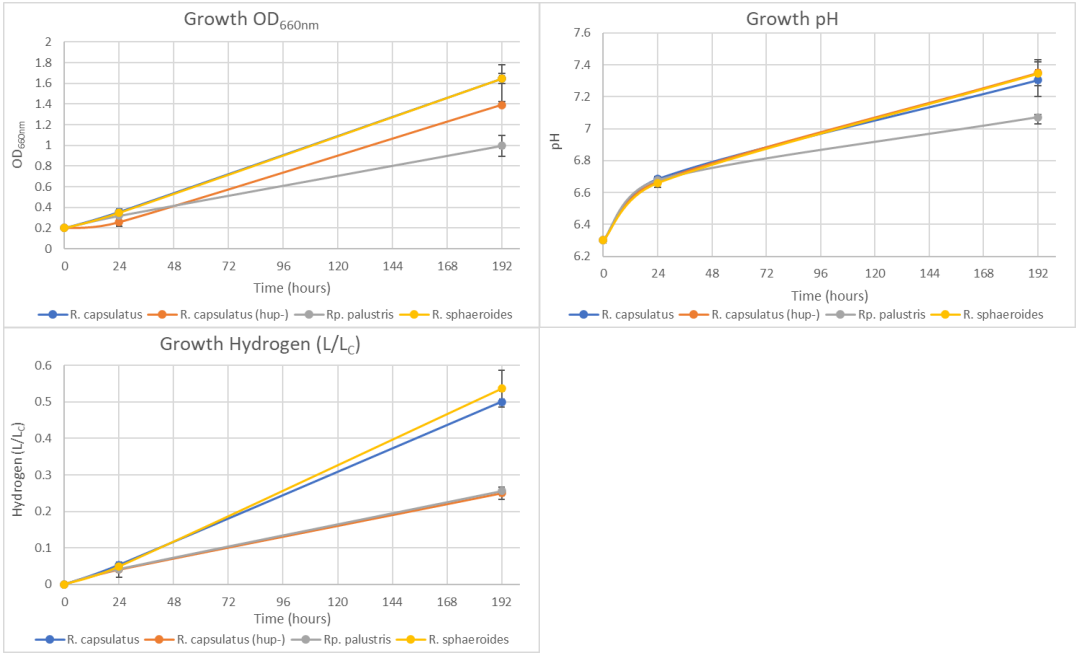

Figure 3.1.1 Growth profile by Optical Density at 660 nm, pH variation and H_2 Production for single-cultures of *R. capsulatus* (DSM 1710), *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in

suspension, cultivated on BP medium supplemented with 20 mM Acetate/10 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux during the first growth from the stock.

3.2 Experiments with Previously Designed 1.4 L Plexiglass Panel Photobioreactors

Photobioreactor is the key equipment for biological hydrogen production. It provides a convenient environment for the growth and survival of the photosynthetic organisms during hydrogen production.

Therefore, the selection and the design of photobioreactors is important. A variety of photobioreactors have been designed and constructed for hydrogen production for suspended cultures. However, immobilization of cells offers many advantages over suspended cultures and allows smaller reactor volumes compared to suspended cultures.

Different types of immobilized photobioreactors were built for both indoor and outdoor experiments in our laboratories (Elkahlout *et al.,* 2018, Sagir *et al.,* 2018).

Among those reactors, a 1.4 L plexiglass panel reactor type was chosen, and first immobilization studies were conducted with those reactors. pH and Hydrogen production data were acquired. Single-cultures of and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) were experimented in outdoors. *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) were selected, due to their observed synergistic hydrogen production performances in suspended cultures (Baysal, 2012). Results are given in Figure 3.2.1.

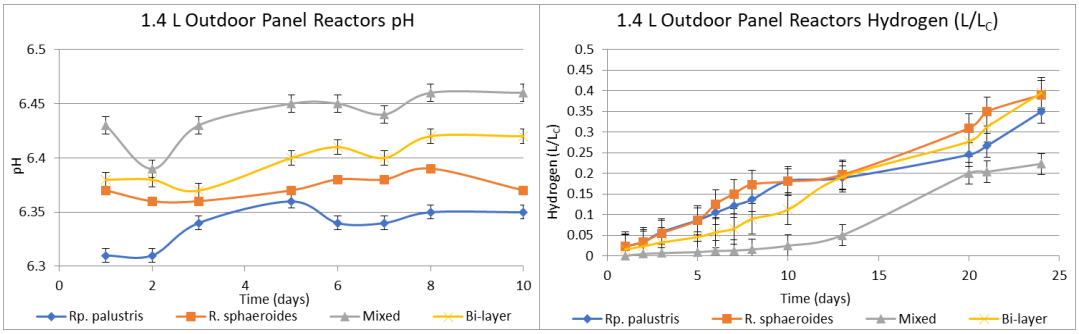

Figure 3.2.1 pH variation and H_2 Production for 1.4 L single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight outdoors.

The trials with the 1.4 L reactor type did not provide satisfactory hydrogen production performances. For single cultures and the co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864). Suspended culture studies have yielded between 1.48 to 1.77 L/L$_C$ hydrogen production, 0.51 to 0.94 mmol/L.h hydrogen productivities, 1.65 to 2.23 mol hydrogen / mol acetate hydrogen yields and 41.32 to 55.64 % substrate conversion efficiencies (Baysal, 2012). Results of the 1.4 L reactors trials are given in Table 3.2.1.

Table 3.2.1 Total Hydrogen Produced (L/L_C), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h), Substrate Conversion Efficiency (%) and Final pH comparison of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 1.4 L reactors, outdoors.

	Total Hydrogen Produced	Hydrogen Yield	Hydrogen Productivity	Substrate Conversion Efficiency	pH
Rp. palustris Outdoor	0.350	0.390	0.027	9.765	6.35
R. sphaeroides Outdoor	0.389	0.434	0.030	10.856	6.37
Mixed Outdoor	0.222	0.248	0.017	6.214	6.46
Bi-layer Outdoor	0.395	0.441	0.030	11.033	6.42

1.4 L Panel Reactors were observed to suffer from complications. Assembly of the reactors themselves required various plexiglass and plastic parts -all of which are prone to cracking and leaking- to be screwed together -which is as a technique, also prone to leaking- with rubber sheets -which are prone to tearing, and again leaking- in between. The low hydrogen production performances observed might be caused of gas leakage inside and hydrogen leakage outside. pH values were within normal range, however did not increase as expected. This might be due to oxygenation and lack of sufficient hydrogen production metabolism due to leakages.

Additionally, a synergistic effect of using co-cultures of PNSB was not observed. Bi-layer co-culture configuration has yielded only marginally better performances than the single cultures, and the mixed co-culture configuration has performed much lower.

3.3 Experiments with 300 mL Plexiglass Cell Culture Flasks

As a simpler reactor model, 300 mL Plexiglass Cell Culture Flasks were utilized for next immobilization experiments. For the first runs, pH and Hydrogen production data were acquired. Single-cultures of and mixed and bi-layer co-cultures of *Rp.*

palustris (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) were experimented in indoors and outdoors. Results are given in Figures 3.3.1 and 3.3.2.

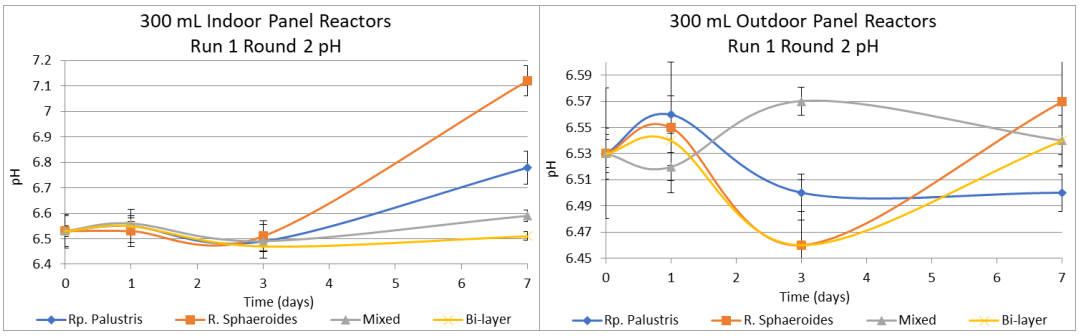

Figure 3.3.1 pH variation for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. Run 1.

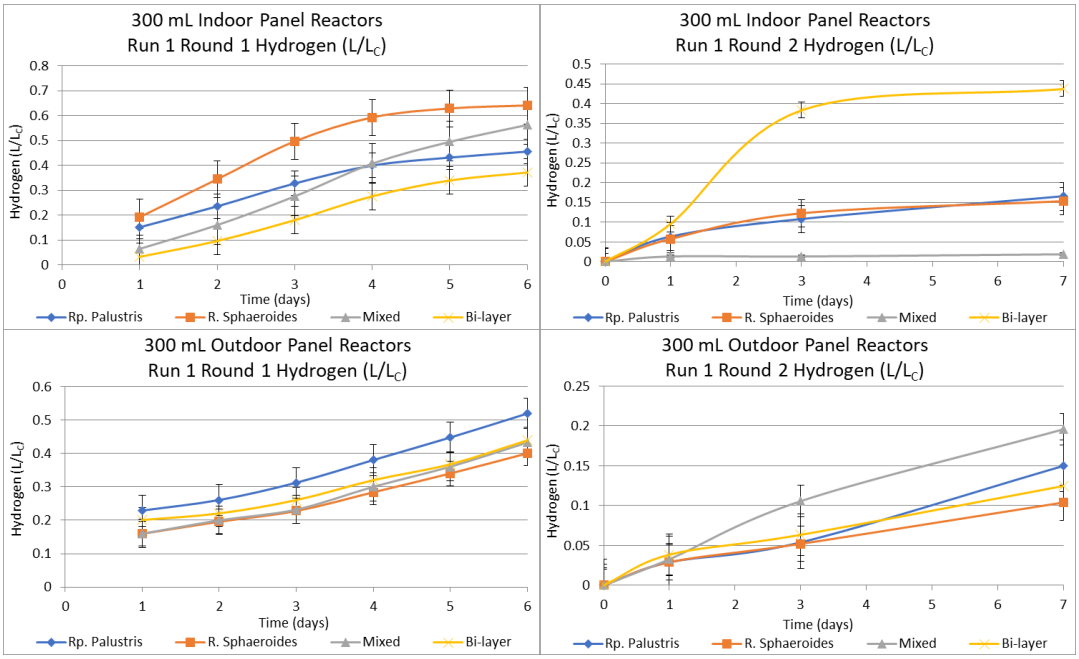

Figure 3.3.2 H_2 Production for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. Run 1.

As given in Tables 3.3.1 and 3.3.2, the first trials with the 300 mL reactor type have yielded much better performances than the trials with the 1.4 L reactor for the first round. However, the performances were still not on par with the suspension culture results. In the second round, performances decreased drastically. 300 mL reactors were found out to be prone to cracking from the junctions on the edges. Although produced gas is transferred to gas collection system, the internal pressure accumulated during the experiments caused cracks. Results show that, higher the performance for an experimental group was in the first round, lower for the second round. Which might be due to the cracks formed due to the higher internal pressure for the better performing groups. As seen in the Figure 3.3.2, the outdoor groups have performed better on the first day, however their performances decreased afterwards. Which might be again, due to crack formations as a result of higher

performance. pH values were within the expected range, however did not increase as expected, and unexpected fluctuations were observed for the outdoor experiments, which might be due to the gas and liquid transfer due to the cracks.

Table 3.3.1 Total Hydrogen Produced (L/L_C), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h) and Substrate Conversion Efficiency (%) comparison of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 300 mL reactors, indoors and outdoors. Run 1. Round 1.

	Total Hydrogen Produced	Hydrogen Yield	Hydrogen Productivity	Substrate Conversion Efficiency
Rp. palustris Indoor	0.456	0.509	0.141	12.723
R. sphaeroides Indoor	0.640	0.714	0.198	17.857
Mixed Indoor	0.564	0.629	0.175	15.737
Bi-layer Indoor	0.372	0.415	0.115	10.379
Rp. palustris Outdoor	0.520	0.580	0.161	14.509
R. sphaeroides Outdoor	0.128	0.143	0.040	3.571
Mixed Outdoor	0.432	0.482	0.134	12.054
Bi-layer Outdoor	0.440	0.491	0.136	12.277

Table 3.3.2 Total Hydrogen Produced (L/L_C), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h), Substrate Conversion Efficiency (%) and Final pH comparison of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 300 mL reactors, indoors and outdoors. Run 1. Round 2.

	Total Hydrogen Produced	Hydrogen Yield	Hydrogen Productivity	Substrate Conversion Efficiency	pH
Rp. palustris Indoor	0.165	0.184	0.044	4.607	6.78
R. sphaeroides Indoor	0.154	0.171	0.041	4.286	7.12
Mixed Indoor	0.019	0.021	0.005	0.536	6.59
Bi-layer Indoor	0.044	0.049	0.012	1.232	6.51
Rp. palustris Outdoor	0.150	0.167	0.040	4.179	6.5
R. sphaeroides Outdoor	0.104	0.116	0.028	2.893	6.57
Mixed Outdoor	0.196	0.219	0.052	5.464	6.54
Bi-layer Outdoor	0.125	0.139	0.033	3.482	6.54

Additionally, when the effect of co-cultivation is analyzed, higher performance than the single cultures were observed just for the mixed outdoor group of the second round, which had inadequate performances compared to the first round and the suspension culture results, a reliable interpretation might not be made.

Next, another set of experiments were performed with 300 mL reactors. This time, the reactors were supported from the junction edges by use of cyanoacrylamide before beginning the experiments. After the first round, new cracks were observed

and hence, during the rest of the rounds, the reactors were checked daily against newly formed cracks and fixed as new cracks were observed. pH, hydrogen production, and acetate consumption profiles are given in Figures 3.3.3, 3.3.4, 3.3.5, 3.3.6, 3.3.7 and 3.3.8.

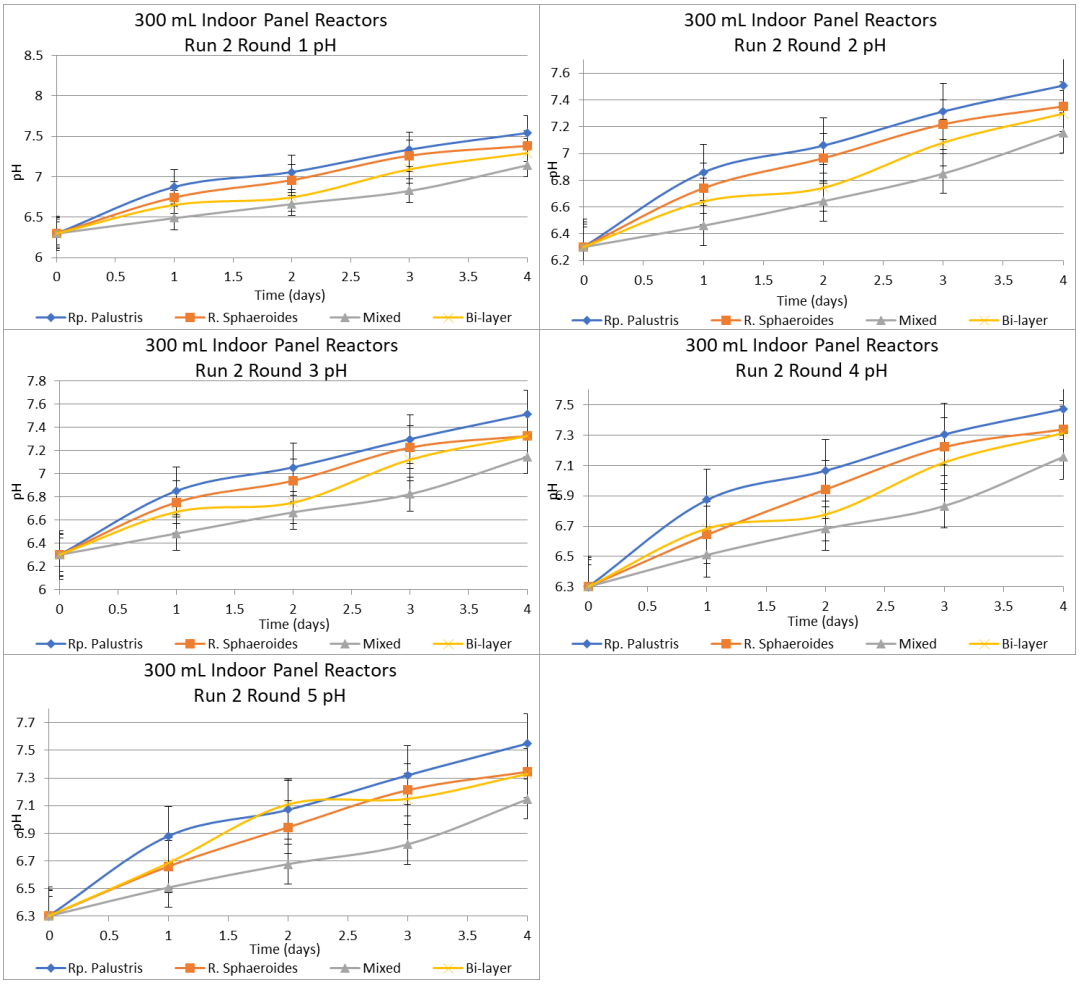

Figure 3.3.3 pH variation for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux. Run 2.

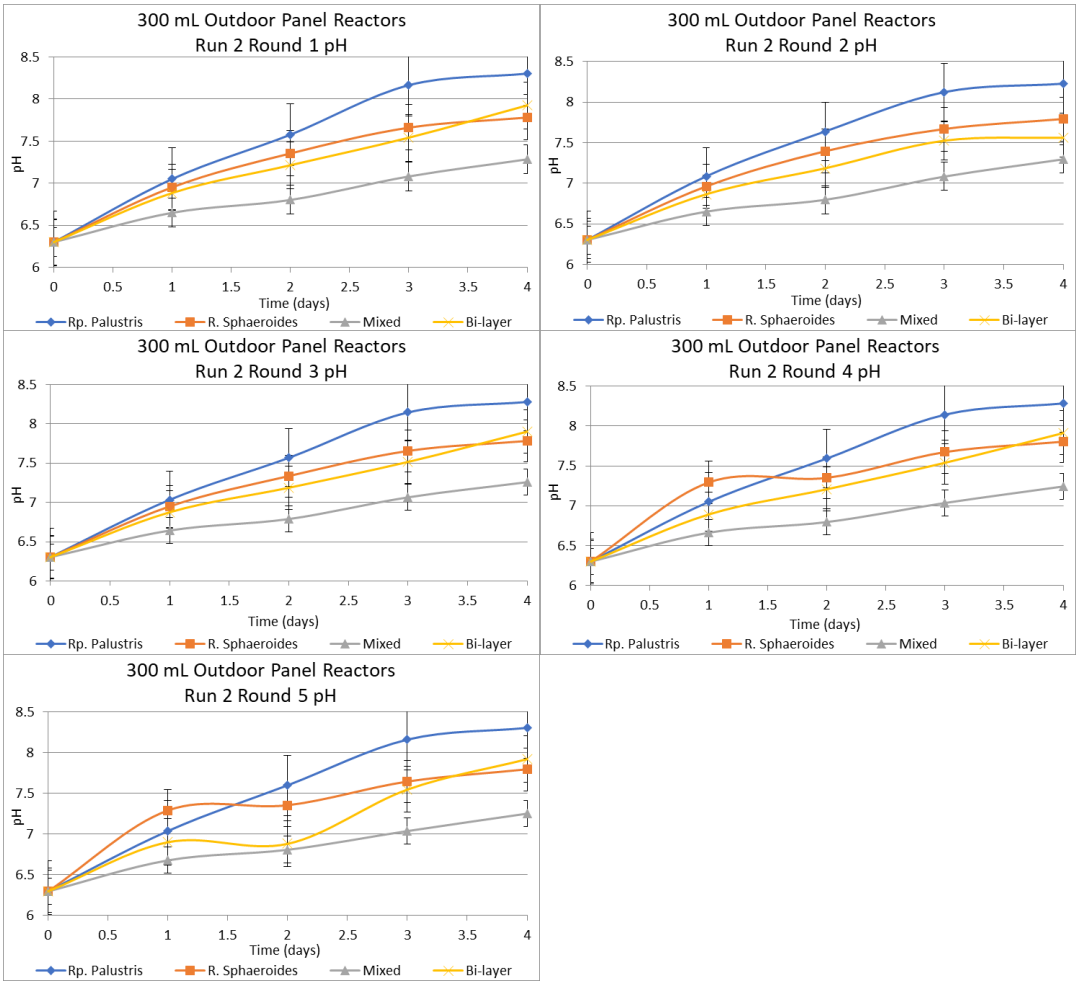

Figure 3.3.4 pH variation for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight. Run 2.

On this run, pH values increased as expected, and stayed within the expected range.

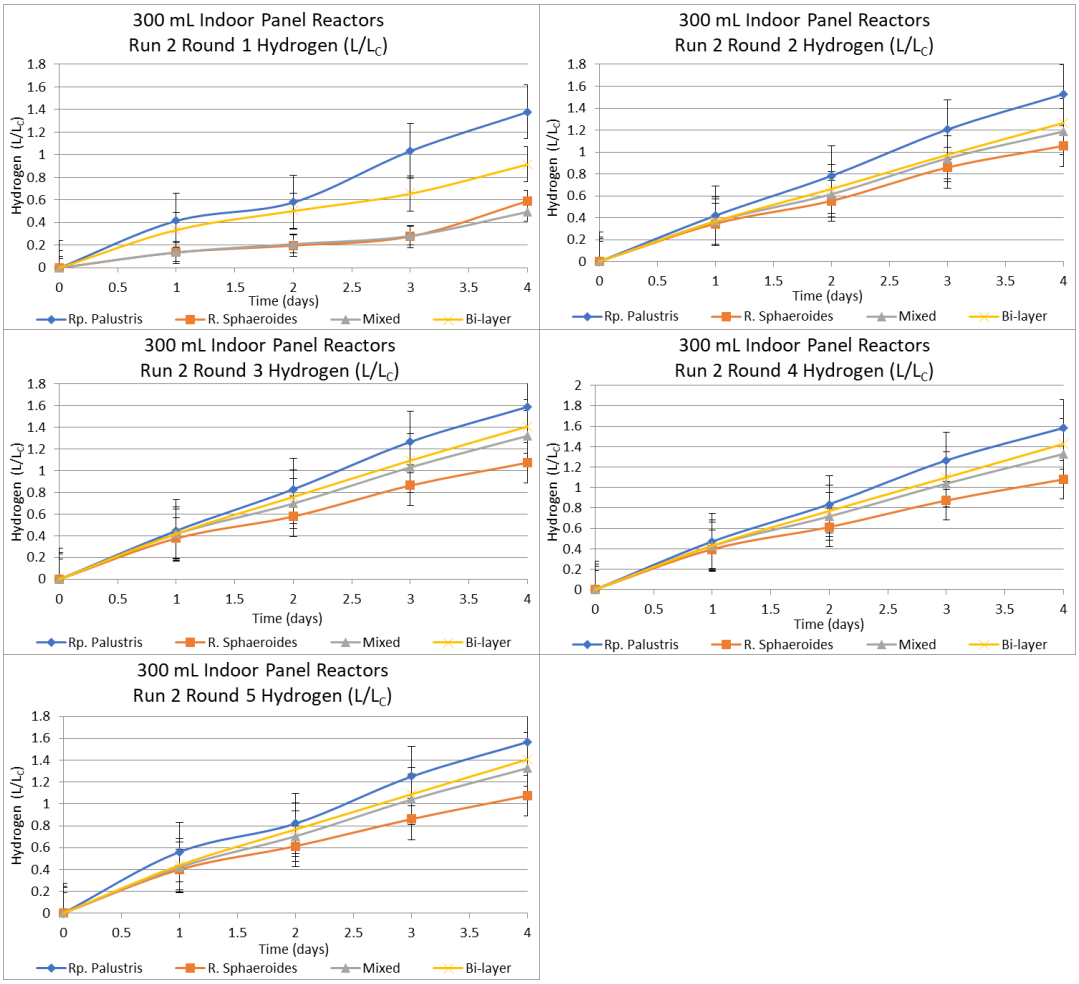

Figure 3.3.5 H_2 Production for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500. Run 2.

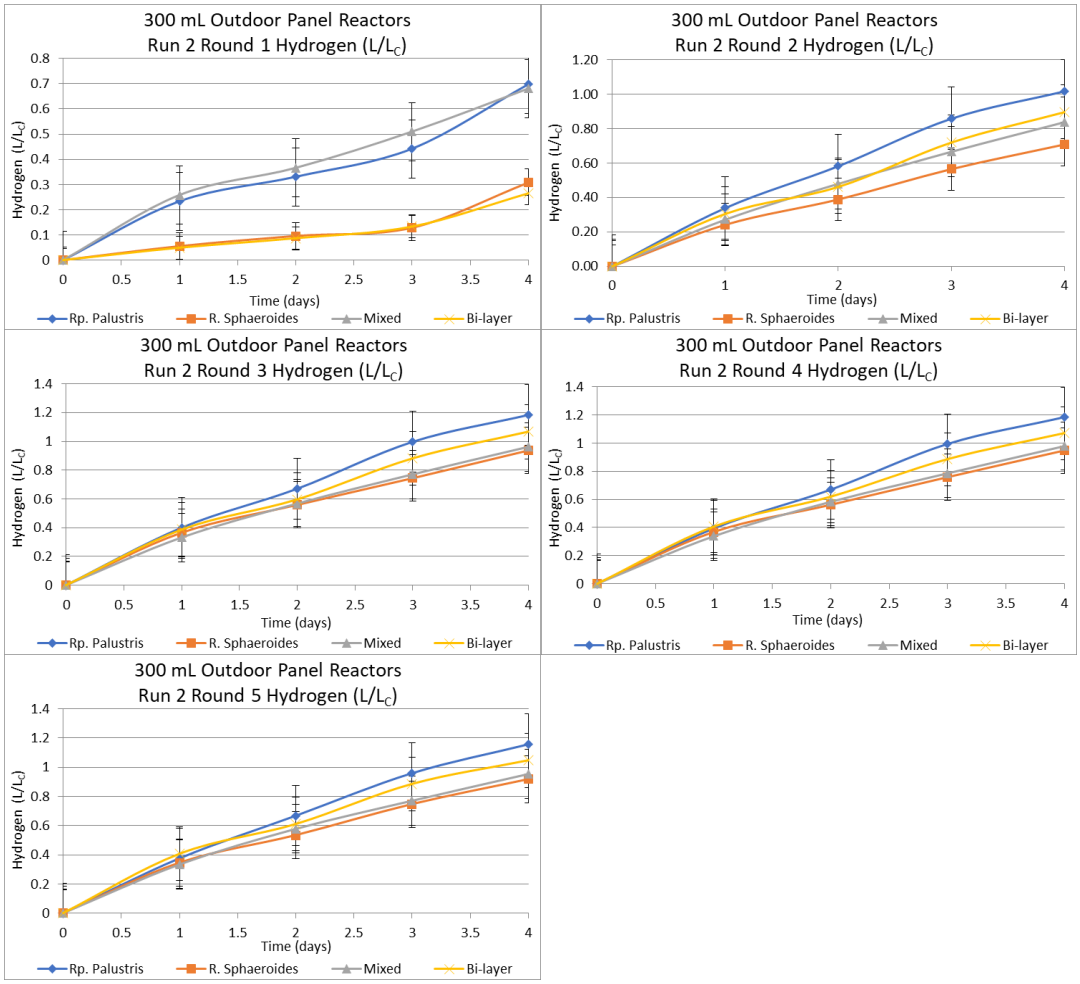

Figure 3.3.6 H_2 Production for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight. Run 2.

Hydrogen production performances were observed to be improved drastically, except for the first round, which might be due to cracks. However, the performances were still lower than the suspended culture results. Results show that, *Rp. palustris* (DSMZ 127) has exhibited the highest and *R. sphaeroides* O.U.001 (DSMZ 5864) has exhibited the lowest performances, and the co-cultures has resulted in

performances in between. Bi-layer co-culture configuration seemed to yield better results than the mixed co-culture, however, a performance increase was not observed over *Rp. palustris* (DSMZ 127). The bi-layer gel surface was prepared for these reactors by application of the agar-culture of one strain by pouring, then after solidification, application of the other strain. Since the volume required to solidify in each step is decreased, tjhe solidification duration of the gel was also decreased, and the bacteria were hence subjected to lower heat stress. The difference between the mixed and the bi-layer co-cultures might be a result of that fact.

Additionally, indoor groups have yielded better performances than the outdoor groups. Gel-bacteria mixtures of the outdoor groups were found to be bleached out by time. High light intensity during mid-time might have caused photo-bleaching and might have affected hydrogen production performances.

Apart from the first round, which was suffered due to leakages, all other rounds have resulted in similar hydrogen production performances. Immobilization might have ensured multiple use of culture with a reliable performance profile.

All experimented groups were observed to consume their substrate within 4 days, as given in Figures 3.3.7 and 3.3.8 below.

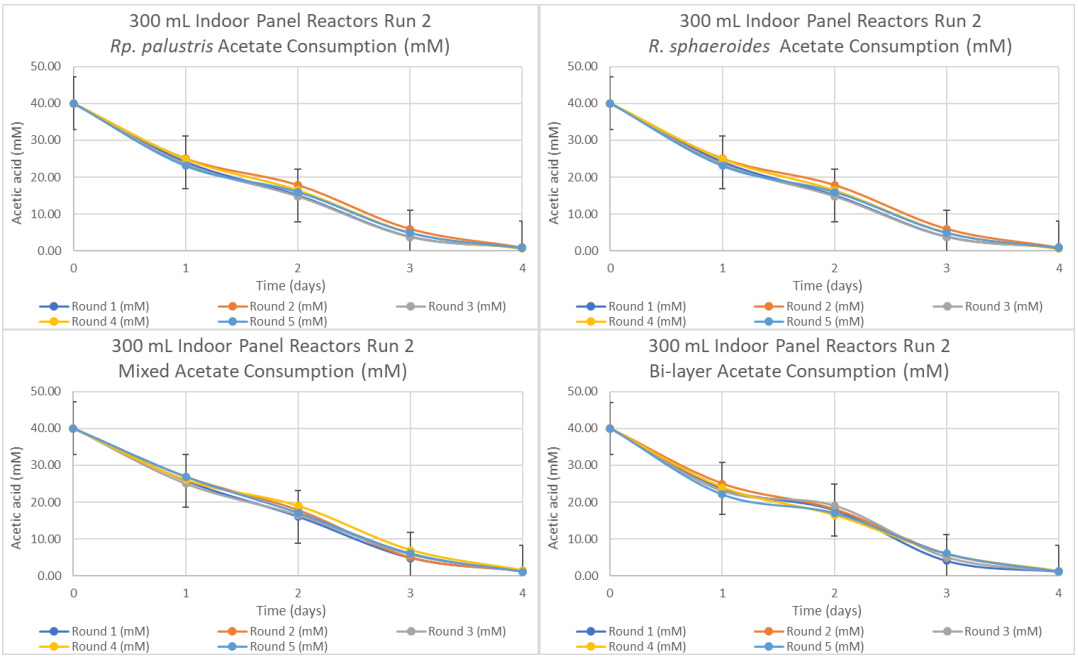

Figure 3.3.7 Acetate Consumption for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux. Run 2.

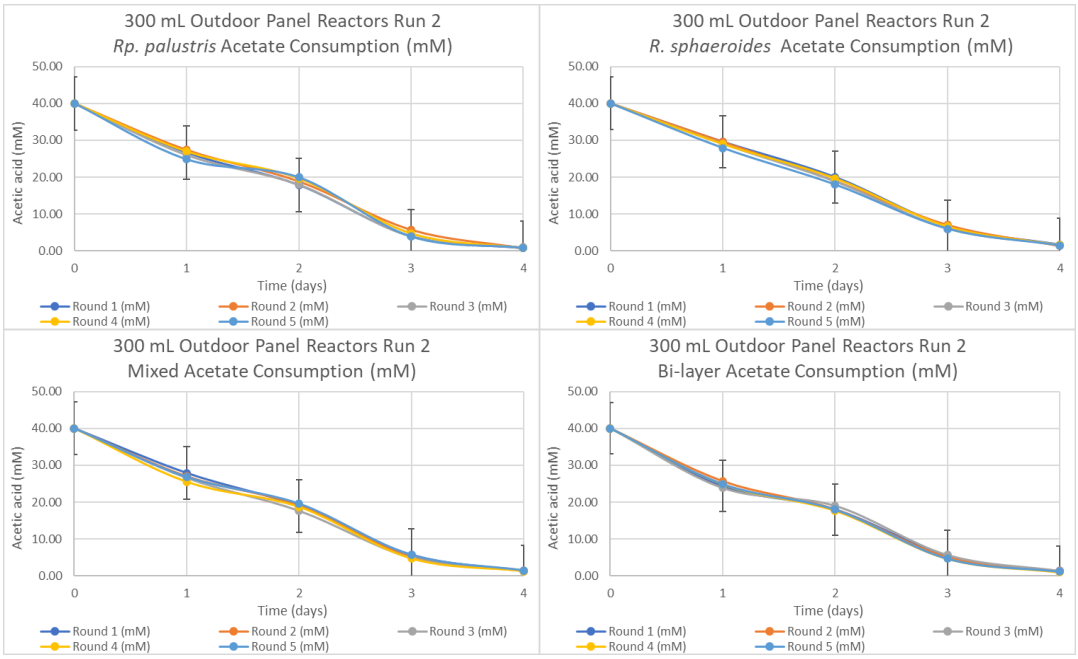

Figure 3.3.8 Acetate Consumption for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight. Run 2.

pH variation of all rounds is given in Figure 3.3.9 below. Similar profile for all rounds were observed.

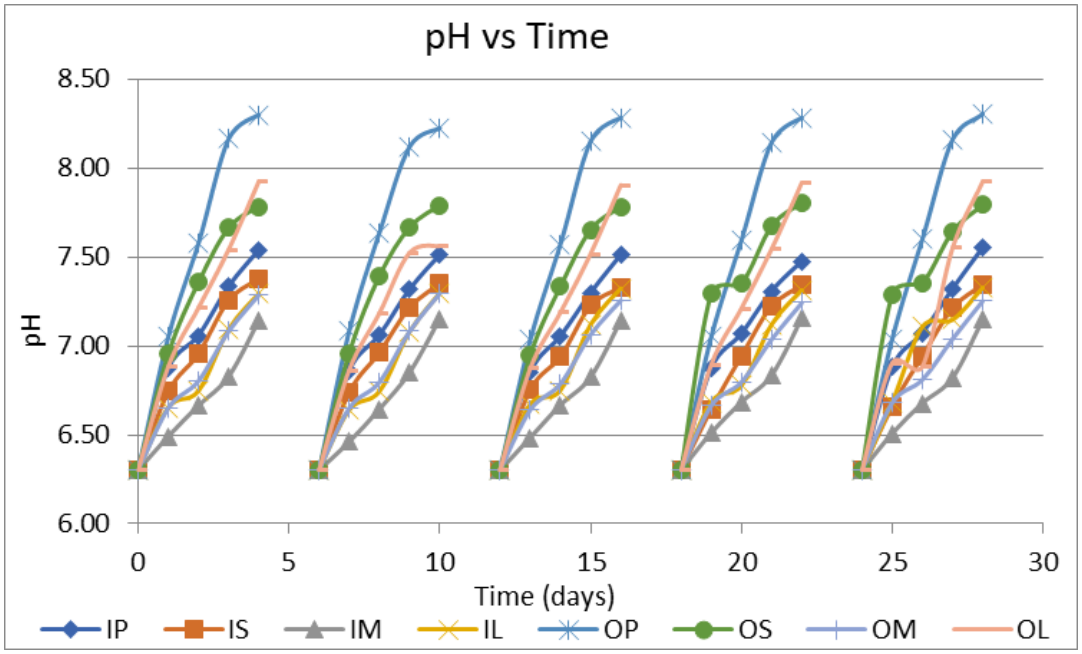

Figure 3.3.9 pH variation for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) M: Mixed L: Bi-layer I: Indoors, O: Outdoors. Run 2.

Hydrogen production in all rounds is given below in Figure 3.3.10. As observed, the performances are comparable during all consecutive rounds, and do not decline by time.

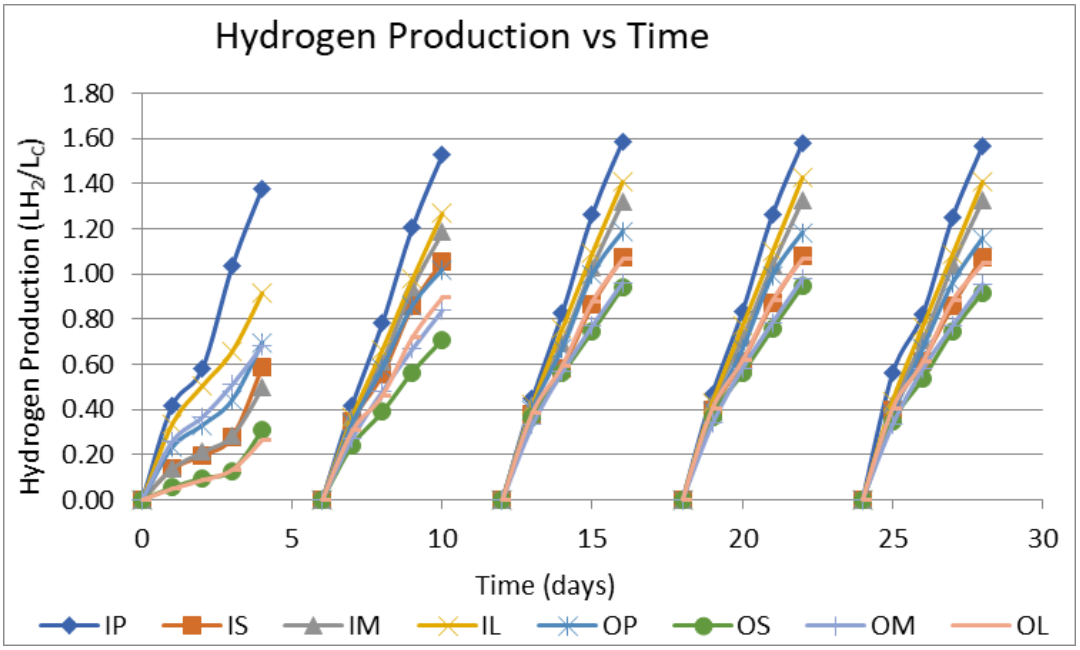

Figure 3.3.10 H_2 Production for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) M: Mixed L: Bi-layer I: Indoors, O: Outdoors. Run 2.

Below in Figure 3.3.11, averages of the final pH values in all rounds are given. *Rp. palustris* (DSMZ 127) has led to highest pH increases, mixed co-cultures have led to lowest increases, and *R. sphaeroides* O.U.001 (DSMZ 5864) and Bi-layer configuration have led to increases in between for both within outdoor and indoor groups. Outdoor groups seem to have caused higher pH increase.

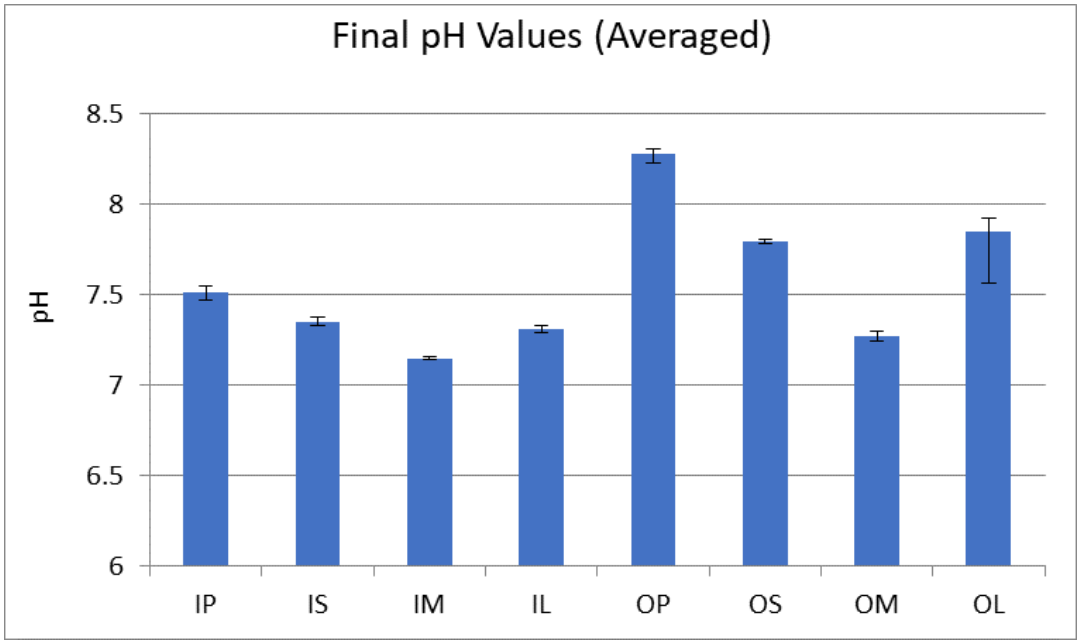

Figure 3.3.11 Final pH values for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) M: Mixed L: Bi-layer I: Indoors, O: Outdoors. Run 2.

Hydrogen production in consecutive rounds is given in Figures 3.3.12 and 3.3.13. As observed, co-cultures have exhibited between the performances of single cultures, and the profiles during the rounds were similar, and did not decline. Note that, in Figure 3.3.12, the hydrogen production is given in mL. Since the reactors are emptied and re-filled between rounds, and the culture media on each round is 200 mL, the culture media increases by 200 mL on each round, hence depicting cumulative hydrogen production in L/L_C would be misleading.

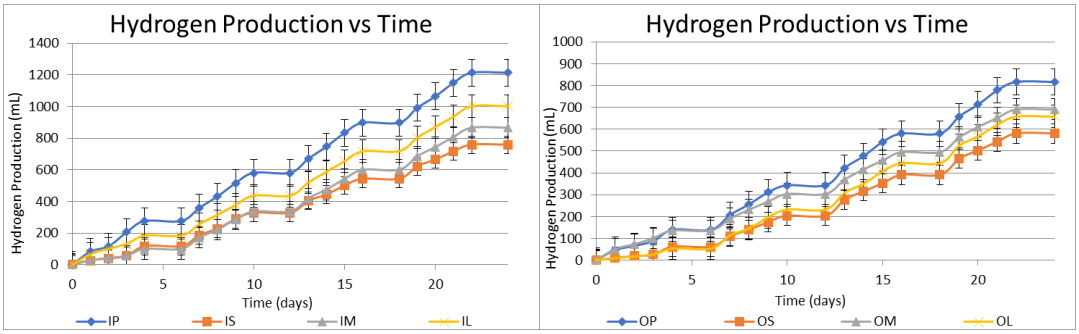

Figure 3.3.12 H_2 Production for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) M: Mixed L: Bi-layer I: Indoors, O: Outdoors. Run 2.

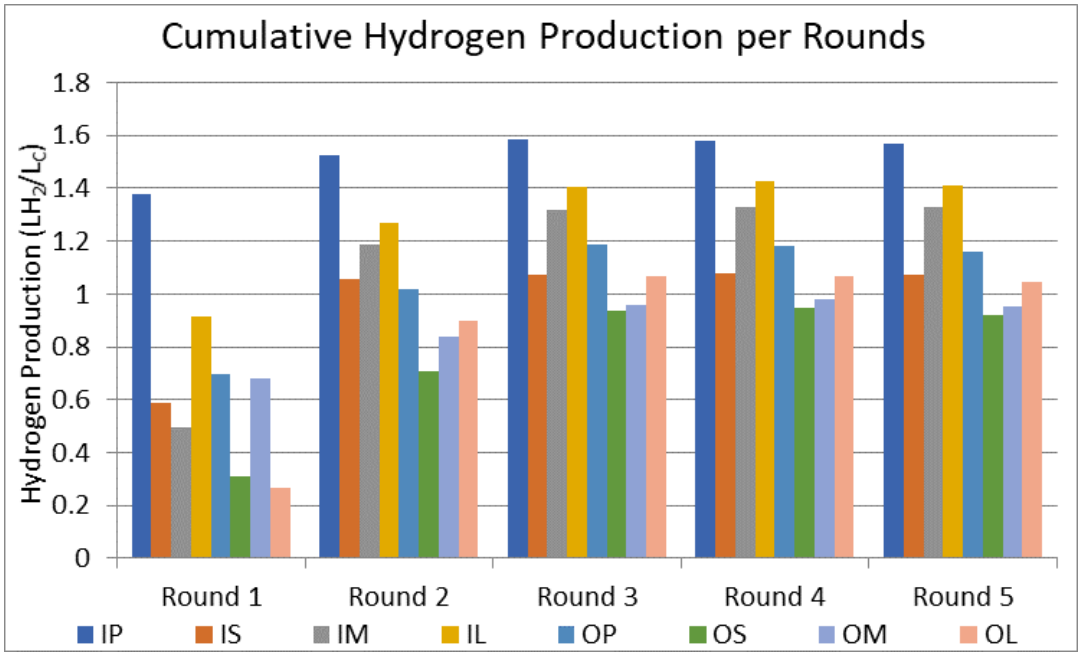

Figure 3.3.13 Cumulative H_2 Production per Rounds for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) M: Mixed L: Bi-layer I: Indoors, O: Outdoors. Run 2.

Cumulative hydrogen production on all rounds is given in Figure 3.3.14. Indoor groups have clearly exhibited better performances.

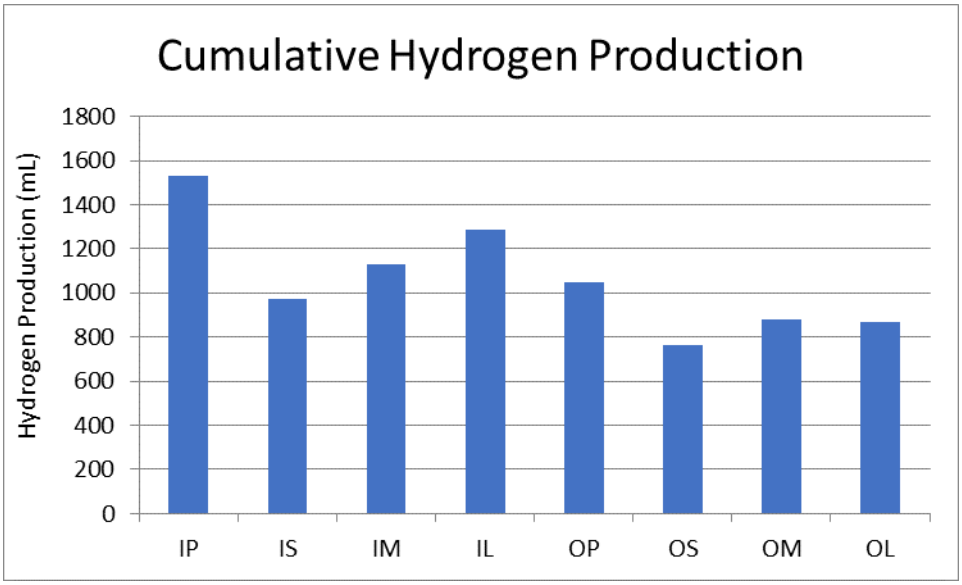

Figure 3.3.14 Cumulative H_2 Production for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) M: Mixed L: Bi-layer I: Indoors, O: Outdoors. Run 2.

Average hydrogen productivities are given in Figure 3.3.15. Results comply with the total hydrogen production results.

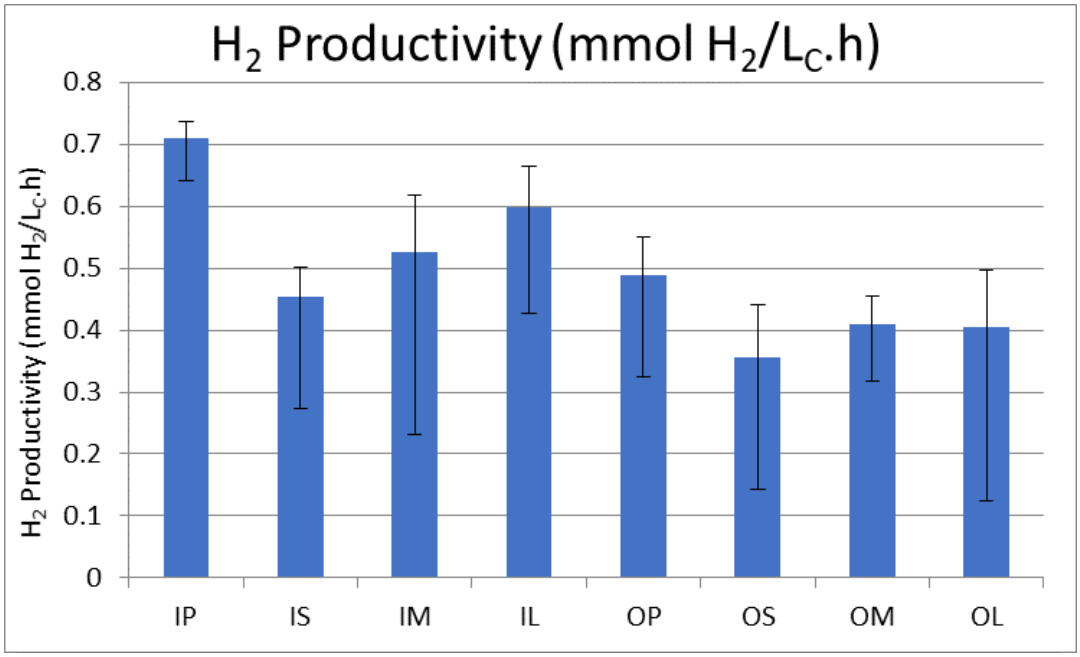

Figure 3.3.15 H_2 Productivity (mmol H_2/L.h) for 300 mL single-cultures and mixed and bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoors and sunlight for outdoors. P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) M: Mixed L: Bi-layer I: Indoors, O: Outdoors. Run 2.

Outdoor temperature and solar radiation measurements are given in Figure 3.3.16. Daily solar radiation fluctuations were low, and a temperature fall was observed during the third and the fourth rounds, however this temperature fluctuation did not seem to affect performances, probably due to the controlled temperature bath used for stabilizing the temperature to 30°C.

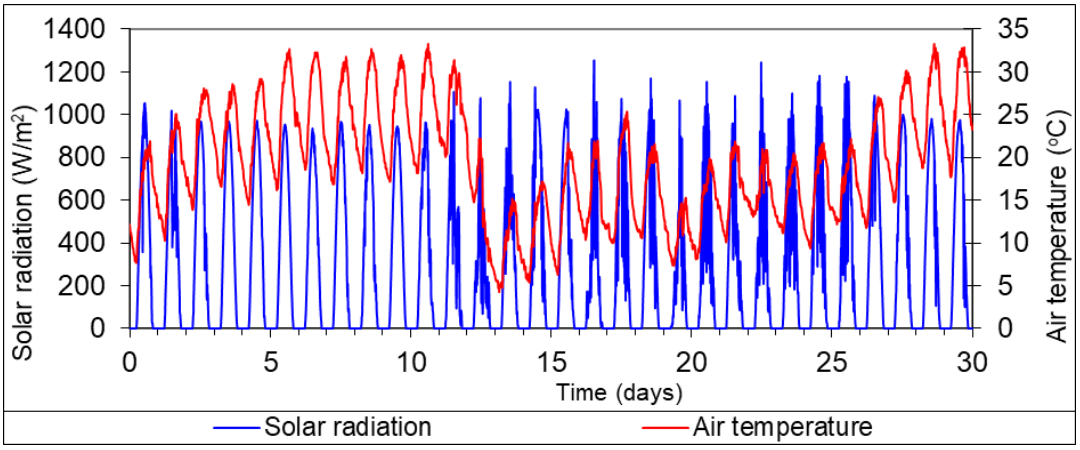

Figure 3.3.16 Daily outdoor temperature and solar radiation measurements taken during the 300 mL outdoor experiments performed between May 10, 2020, and June 8, 2020. Run 2.

Below, in the Table 3.3.3, Productivities of the experimented groups are given. *Rp. palustris* (DSMZ 127) has performed better than suspension cultures and *R. sphaeroides* O.U.001 (DSMZ 5864) has performed comparably. However, Co-cultures performances of the suspension cultures seem to not met. Results comply with hydrogen production profiles.

Table 3.3.3 Productivity (mmol H_2/L.h) comparison of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 300 mL reactors, indoors and outdoors. Run 2.

Productivity	*Rp. palustris*		*R. sphaeroides*		Mixed		Layer by Layer	
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Round 1	0.642	0.324	0.273	0.143	0.231	0.316	0.426	0.124
Round 2	0.710	0.473	0.491	0.329	0.551	0.390	0.590	0.417
Round 3	0.737	0.551	0.499	0.436	0.614	0.447	0.654	0.496
Round 4	0.735	0.550	0.502	0.440	0.618	0.455	0.664	0.497
Round 5	0.729	0.539	0.499	0.427	0.618	0.444	0.655	0.486

Below, in the Table 3.3.4, Hydrogen yields of the experimented groups are given. Performances are again lower than the suspended cultures, and co-cultivation have not yielded better results. Results comply with hydrogen production profiles.

Table 3.3.4 Hydrogen Yield (mol H_2/mol Acetate) comparison of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 300 mL reactors, indoors and outdoors. Run 2.

Molar Yield	*Rp. palustris*		*R. sphaeroides*		Mixed		Layer by Layer	
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Round 1	1.540	0.778	0.656	0.344	0.553	0.759	1.022	0.297
Round 2	1.704	1.136	1.177	0.789	1.322	0.935	1.415	1.001
Round 3	1.769	1.323	1.198	1.047	1.473	1.072	1.570	1.189
Round 4	1.764	1.321	1.204	1.056	1.484	1.092	1.594	1.193
Round 5	1.749	1.293	1.199	1.024	1.483	1.065	1.572	1.168

Below, in the Table 3.3.5, Substrate Conversion Efficiencies of the experimented groups are given. Performances are again lower than the suspended cultures,

although comparable, and co-cultivation have not yielded better results. Results comply with hydrogen production profiles.

Table 3.3.5 Substrate Conversion Efficiency (%) comparison of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 300 mL reactors, indoors and outdoors. Run 2.

Substrate Conversion Efficiency	*Rp. palustris*		*Rp. sphaeroides*		Mixed		Layer by Layer	
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Round 1	38.493	19.447	16.397	8.609	13.836	18.986	25.547	7.414
Round 2	42.597	28.406	29.434	19.728	33.061	23.384	35.382	25.033
Round 3	44.233	33.074	29.940	26.178	36.829	26.802	39.246	29.736
Round 4	44.095	33.023	30.098	26.390	37.089	27.302	39.840	29.833
Round 5	43.723	32.333	29.970	25.605	37.064	26.635	39.2940	29.190

Below, in the Table 3.3.6, the final pH values of the experimented groups are given. Values are closed within each group among different rounds.

Table 3.3.6 Final pH values of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 300 mL reactors, indoors and outdoors. Run 2.

pH	*Rp. palustris*		*R. sphaeroides*		Mixed		Layer by Layer	
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Round 1	7.54	8.30	7.38	7.78	7.14	7.29	7.29	7.92
Round 2	7.51	8.22	7.35	7.79	7.15	7.30	7.30	7.56
Round 3	7.51	8.28	7.33	7.78	7.14	7.26	7.32	7.90
Round 4	7.31	8.28	7.22	7.80	6.83	7.24	7.12	7.92
Round 5	7.55	8.30	7.35	7.79	7.15	7.25	7.33	7.92

Below, in the Table 3.3.7, total hydrogen production for the experimented groups is given. Values are closed within each group among different rounds. Performances are lower than the suspended cultures, and immobilization did not seem to confer advantages. Besides, the higher hydrogen production profiles of co-cultures observed in suspended cultures seem to have been lost.

Table 3.3.7 Total Hydrogen Production (L/L_C) values of single-cultures, and mixed and bi-layer co-cultures of Immobilized PNS Bacteria, experimented in 300 mL reactors, indoors and outdoors. Run 2.

Total H_2 Produced	*Rp. palustris*		*R. sphaeroides*		Mixed		Layer by Layer	
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Round 1	1.380	0.697	0.588	0.309	0.496	0.680	0.916	0.266
Round 2	1.527	1.018	1.055	0.707	1.185	0.838	1.268	0.897
Round 3	1.585	1.185	1.073	0.938	1.320	0.961	1.407	1.066
Round 4	1.580	1.184	1.079	0.946	1.329	0.979	1.428	1.069
Round 5	1.567	1.159	1.074	0.918	1.328	0.955	1.408	1.046

Experiments on 1.4 L reactors have led to inferior results, and switching to the 300 mL plexiglass cell culture flasks have improved hydrogen production drastically. Especially upon detecting the crack formation, and pro-actively taking precautions in the second run, performances have approached desirable levels. However, as stated before, even though the reactors were reinforced beforehand, new cracks continued to form, and an advantage of immobilization nor co-cultivation could not be observed. Hence another reactor type was needed.

3.4 Design of a New Immobilized Photobioreactor Setup

An innovative immobilization method for cylindrical glass bioreactors was used for this work. The transparent body of the reactor in the studies explained above was made of plastic (plexiglass), but glass is more resilient to gas permeation and is more durable. Therefore, glass reactors were picked as the new strategy. Additionally, a cylindrical design for the reactors was selected to provide more consistent exposure to the changing light occurring naturally throughout the day. 150 mL glass reactors were used as reactors. Experiments with 40 and 60 mM initial acetate concentrations, 2500, 4000 lux indoor illumination and sunlight for outdoor experiments were utilized. Since the co-cultivation of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) have not yielded performance gain, single and co-cultures of *R. capsulatus* hup- (YO3) have also been experimented.

Below, in Figure 3.4.1, pH profiles of immobilized bacteria cultivated on BP media with 40 mM acetate under 2500 lux are given. Media was prepared at pH 6.3. Initially, a steep pH rise was observed. After which time, pH tended to decrease for all experimented groups. The first experimental groups which new immobilization technique was tried simultaneously were, 40 mM acetate and 2500 lux illumination for indoors, and 40 mM acetate and sunlight illumination for outdoors groups. While the agar mixture was being poured inside the reactors and let solidify while rotating, relatively high amount of time was consumed. During the preparation of the next experimental groups, empty glass reactors were kept cool in advance to speeding up the solidification process. Bacteria being exposed to higher temperatures during reactor preparation on the first experimental groups hence, might have caused stress on bacteria, and may have shifted their metabolism to accumulation of fatty acids, other metabolites, or growth instead. Which might be the cause of the unexpected pH fall observed. Another possibility would be some unwanted contamination or inclusion of a spoiled ingredient during preparation of the cultures. Still, pH values were within the expected range for all groups.

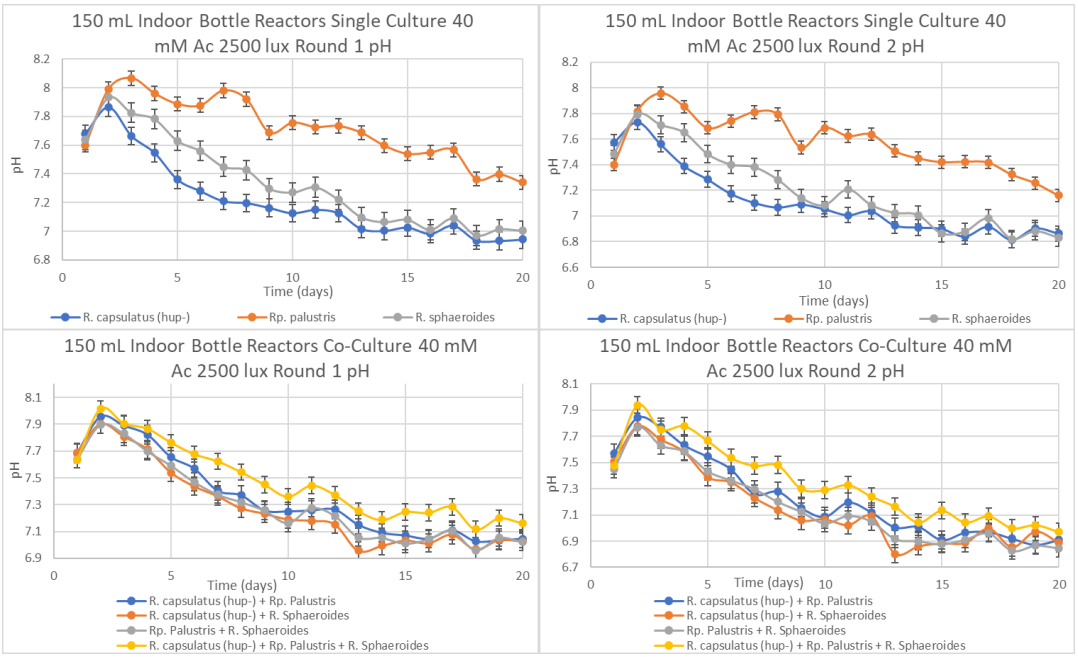

Figure 3.4.1 pH variation for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

Figure 3.4.2 indicates the hydrogen production profiles. *R. capsulatus* hup- (YO3) has yielded highest performances, followed by *R. sphaeroides* O.U.001 (DSMZ 5864) and *Rp. palustris* (DSMZ 127) respectively. Their co-cultures have yielded performances between the performances of the single cultures of their constituent PNSB strains. First round of experiments has yielded higher hydrogen production.

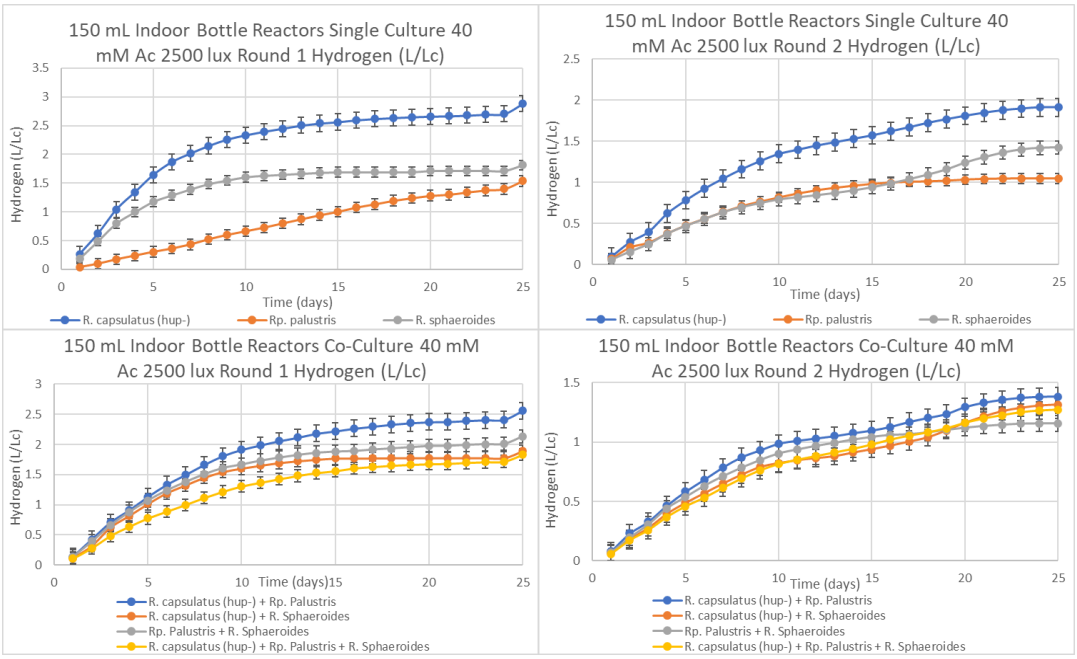

Figure 3.4.2 H_2 Production for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

Figure 3.4.3 reveals the acetic acid utilization of the bacteria. Substrate was consumed within 10 days for all experimental groups. Co-cultures seem to deplete their substrates faster. Although the substrate consumption was completed, experiment was continued, since hydrogen production has continued. Substrate might have been converted to other metabolites, which might have been consumed on the following days.

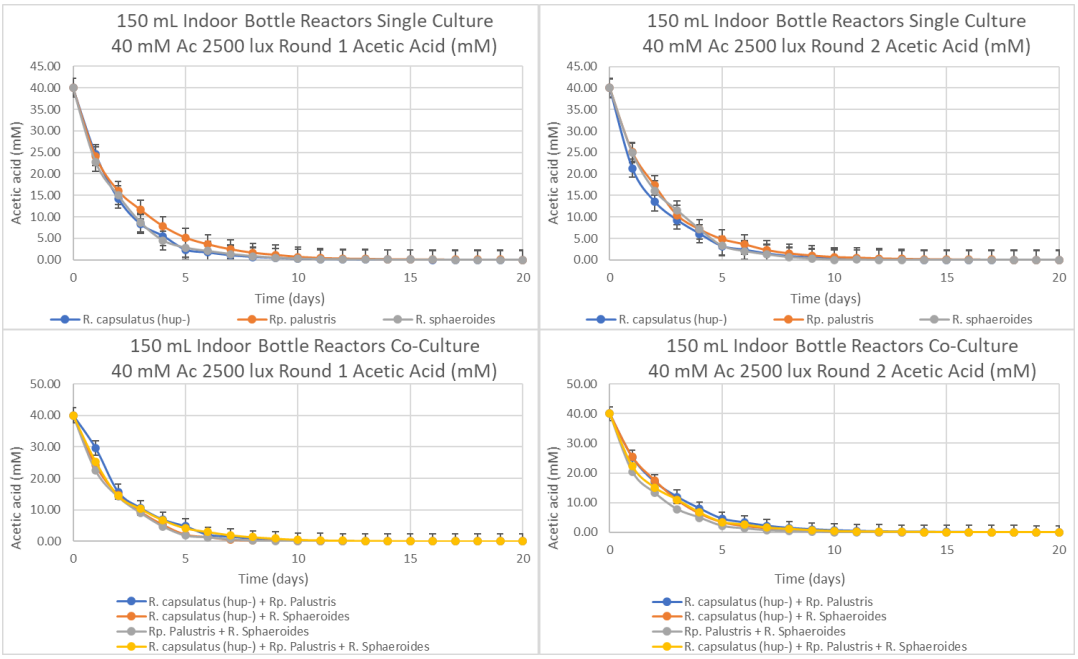

Figure 3.4.3 Acetic acid utilization for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

Tables 3.4.1, 3.4.2, 3.4.3 and 3.4.4 shows total hydrogen produced, hydrogen yield, hydrogen productivity and substrate conversion efficiency of the experimental groups. *R. capsulatus* hup- (YO3) has yielded highest performances among single and co-cultures. Co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) have performed marginally better than single cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) during the first round. The co-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) had the second-best performance after the single culture of *R. capsulatus* hup- (YO3). Single cultures of *R. sphaeroides* O.U.001 (DSMZ 5864) followed. The triple co-cultures have yielded lowest performances. Therefore, an advantage of co-cultivation could not be observed on those experiments. However, this time, higher

hydrogen production, higher yield, and higher substrate conversion efficiencies were observed, in comparison with the single-cultures, especially during the first round (Baysal, 2012).

Table 3.4.1 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h) and Substrate Conversion Efficiency (%) of single-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, indoors. Round 1. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) I: Indoors.

	CI	PI	SI
Total Hydrogen Produced	2.88	1.53	1.81
Hydrogen Yield	3.21	1.71	2.02
Hydrogen Productivity	0.357	0.143	0.224
Subs. Conversion Efficiency	80.4	42.9	50.6

Table 3.4.2 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h) and Substrate Conversion Efficiency (%) of single-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, indoors. Round 2. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) I: Indoors.

	CI	PI	SI
Total Hydrogen Produced	1.86	0.361	1.28
Hydrogen Yield	2.08	0.403	1.43
Hydrogen Productivity	0.173	0.0447	0.159
Subs. Conversion Efficiency	52.1	10.0	35.9

Table 3.4.3 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H2/mol Acetate), Hydrogen Productivity (mmol H2/L.h) and Substrate Conversion Efficiency (%) of co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, indoors. Round 1. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) I: Indoors.

	CPI	CSI	PSI	CPSI
Total Hydrogen Produced	2.56	1.89	2.13	1.83
Hydrogen Yield	2.85	2.11	2.38	2.04
Hydrogen Productivity	0.238	0.175	0.198	0.170
Subs. Conversion Efficiency	71.4	52.7	59.5	51.0

Table 3.4.4 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h) and Substrate Conversion Efficiency (%) of co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, indoors. Round 2. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) I: Indoors.

	CPI	CSI	PSI	CPSI
Total Hydrogen Produced	1.33	1.19	1.23	0.886
Hydrogen Yield	1.48	1.32	1.38	0.988
Hydrogen Productivity	0.123	0.110	0.115	0.0824
Subs. Conversion Efficiency	37.1	33.2	34.5	24.7

40 mM Acetate concentration was also experimented outdoors. Below, the pH variations are given in Figure 3.4.4. pH profiles were coherent with the 40 mM

acetate 2500 lux indoor group, given above. However, for the outdoor groups, initial pH rises from pH 6.3 was observed to be steeper. Then the pH fall is observed similarly.

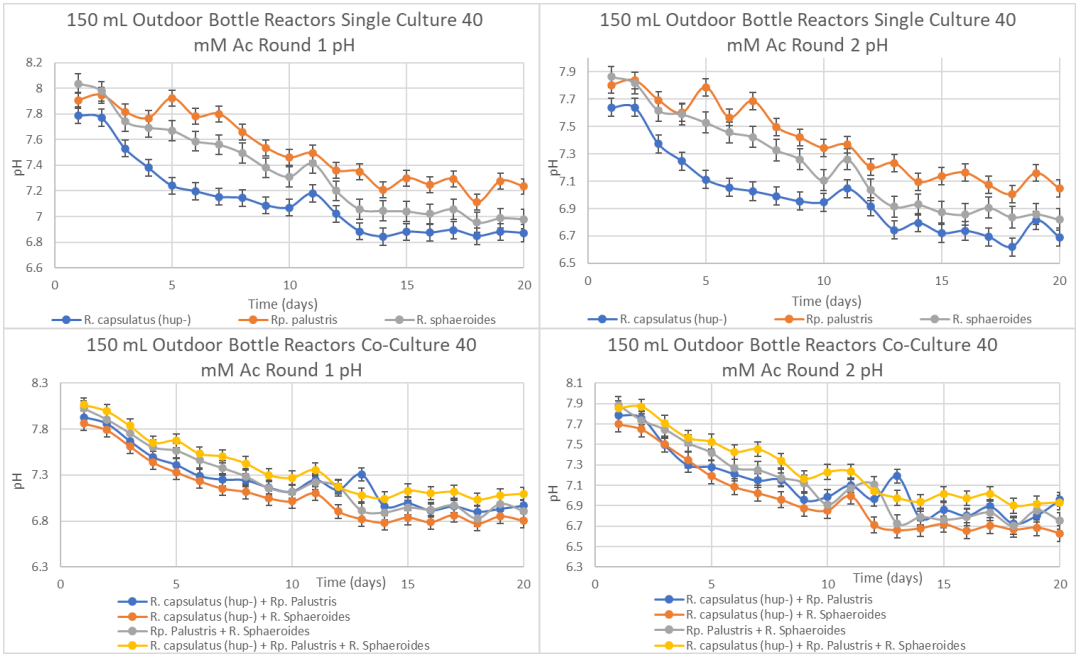

Figure 3.4.4 pH variation for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight.

Hydrogen production profiles are given in Figure 3.4.5. Performances of the experimental groups are parallel to the previous single-culture experiments. Single cultures of *R. capsulatus* hup- (YO3) have yielded highest performances and co-cultures have yielded performances between the single culture performances of their constituent strains. Also, all experimental groups have yielded better performances on Round 1, in comparison to Round 2. However, in general, outdoor cultivation has

yielded higher performance with respect to indoor cultivation. This result has not been observed for the 300 mL reactor experiments.

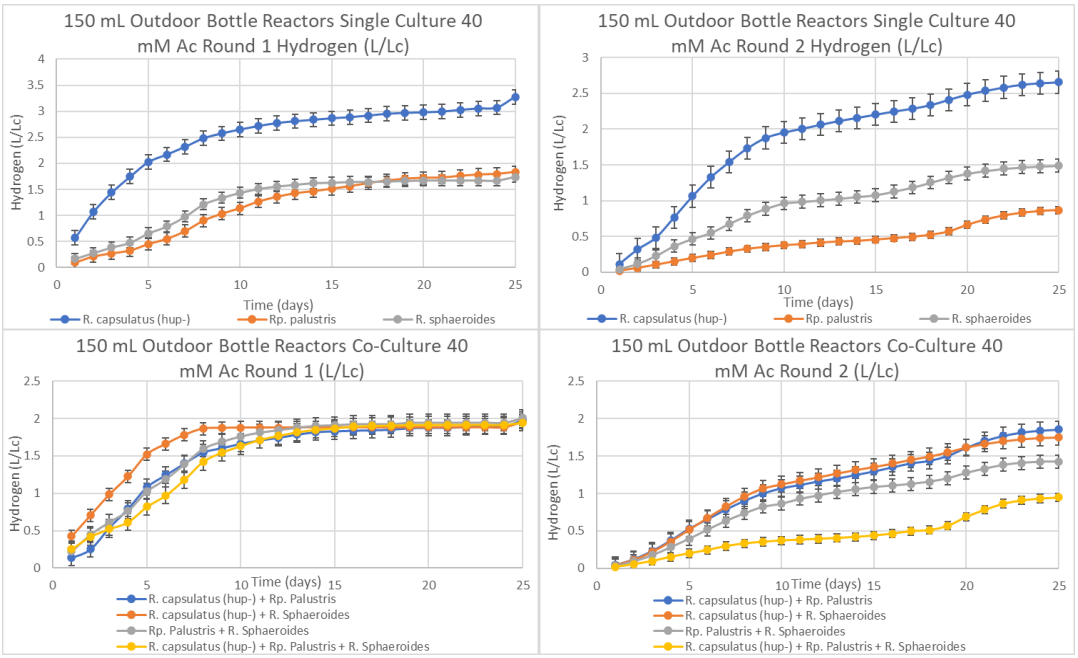

Figure 3.4.5 H_2 Production for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight.

Acetic acid utilization is given in Figure 3.4.6. Substrate consumption profiles were found similar to the indoor experiments. Substrates were consumed within 5-7 days. Again, substrates were consumed faster by the co-cultures. Although the substrate consumption was completed, experiment was continued, since hydrogen production has continued. Substrate might have been converted to other metabolites, which might have been consumed on the following days.

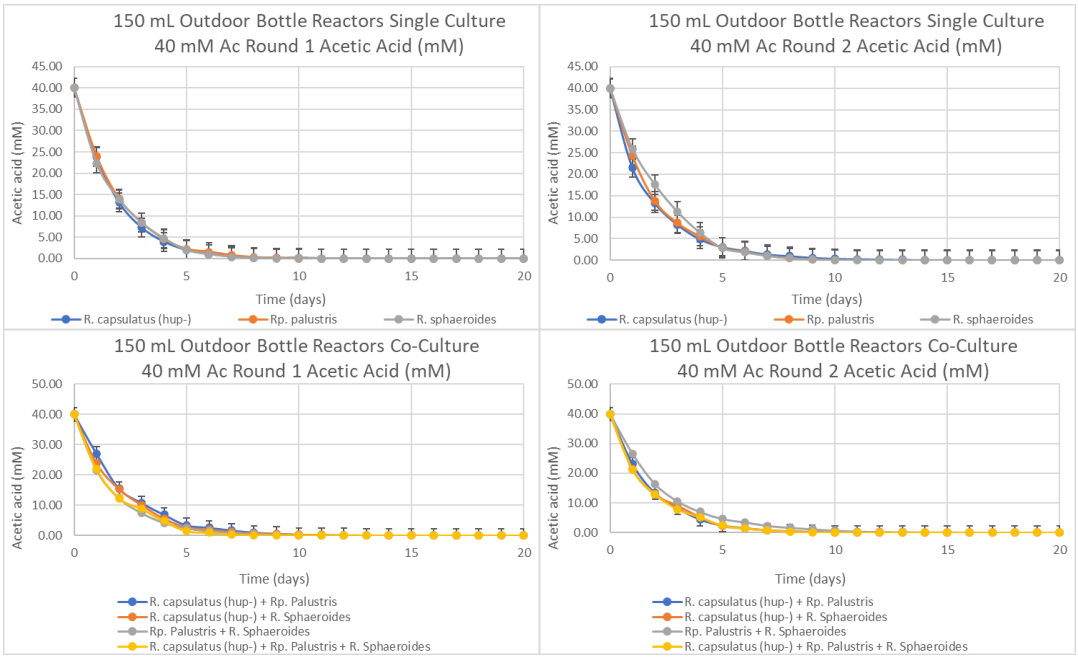

Figure 3.4.6 Acetic acid utilization for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight.

Below, temperature and solar radiation data are given in Figure 3.4.7. Although the season was approaching the fall, and outdoor temperature and solar radiation was decreasing day by day, and outdoor night temperatures below 0°C was experienced, hydrogen production by sunlight utilization could be performed, thanks to the controlled temperature system used for the reactors.

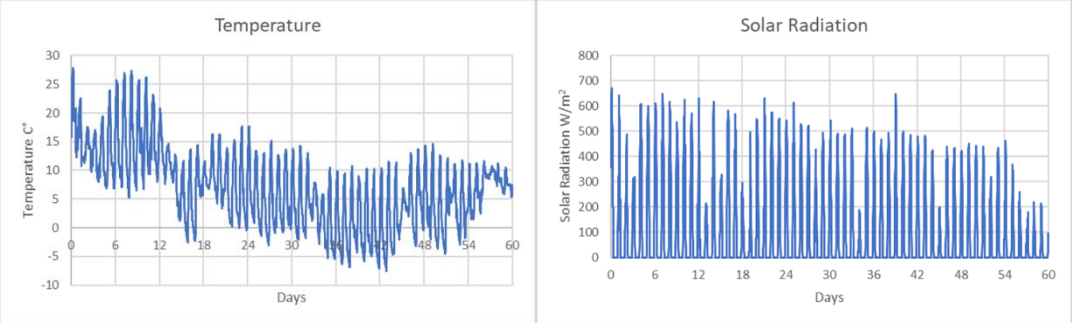

Figure 3.4.7 Daily outdoor temperature and solar radiation measurements taken during the 150 mL outdoor experiments performed between October 17, 2020, and December 15, 2020.

Tables 3.4.5, 3.4.6, 3.4.7 and 3.4.8 shows total hydrogen produced, hydrogen yield, hydrogen productivity and substrate conversion efficiency of the experimental groups. Results comply with the results of the indoor trial with 40 mM acetate under 2500 lux above. However, at this time, the performance increase observed in comparison with the indoor trials above, and the suspension cultures (Baysal, 2012) were higher, especially in the first round. Additionally, during the first round, all co-culture groups have exhibited similar performances. In the second round, the performances decreased even more drastically, especially for the single cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864).

Table 3.4.5 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H2/mol Acetate), Hydrogen Productivity (mmol H2/L.h) and Substrate Conversion Efficiency (%) of single cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, outdoors. Round 1. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) O: Outdoors.

	CO	PO	SO
Total Hydrogen Produced	3.27	1.83	1.74
Hydrogen Yield	3.65	2.04	1.94
Hydrogen Productivity	0.406	0.170	0.161
Subs. Conversion Efficiency	91.3	51.1	48.5

Table 3.4.6 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H2/mol Acetate), Hydrogen Productivity (mmol H2/L.h) and Substrate Conversion Efficiency (%) of single cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, outdoors. Round 2. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) O: Outdoors.

	CO	PO	SO
Total Hydrogen Produced	2.16	0.552	0.788
Hydrogen Yield	2.42	0.616	0.880
Hydrogen Productivity	0.201	0.0514	0.0733
Subs. Conversion Efficiency	60.5	15.4	22.0

Table 3.4.7 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H2/mol Acetate), Hydrogen Productivity (mmol H2/L.h) and Substrate Conversion Efficiency (%) of co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, outdoors. Round 1. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) O: Outdoors.

	CPO	CSO	PSO	CPSO
Total Hydrogen Produced	1.97	1.95	2.00	1.94
Hydrogen Yield	2.20	2.17	2.24	2.17
Hydrogen Productivity	0.245	0.242	0.249	0.241
Subs. Conversion Efficiency	55.1	54.4	56.0	54.3

Table 3.4.8 Total Hydrogen Produced (L/Lc), Hydrogen Yield (mol H2/mol Acetate), Hydrogen Productivity (mmol H2/L.h) and Substrate Conversion Efficiency (%) of co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors, outdoors. Round 2. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864) O: Outdoors.

	CPO	CSO	PSO	CPSO
Total Hydrogen Produced	1.24	1.65	1.18	0.969
Hydrogen Yield	1.38	1.85	1.32	1.08
Hydrogen Productivity	0.115	0.154	0.110	0.0901
Subs. Conversion Efficiency	34.7	46.2	33.1	27.0

In the Figure 3.4.8, minimum and maximum pH ranges are given, starting from the 24th hour after beginning the experimentations. pH ranges were found to be within desirable levels.

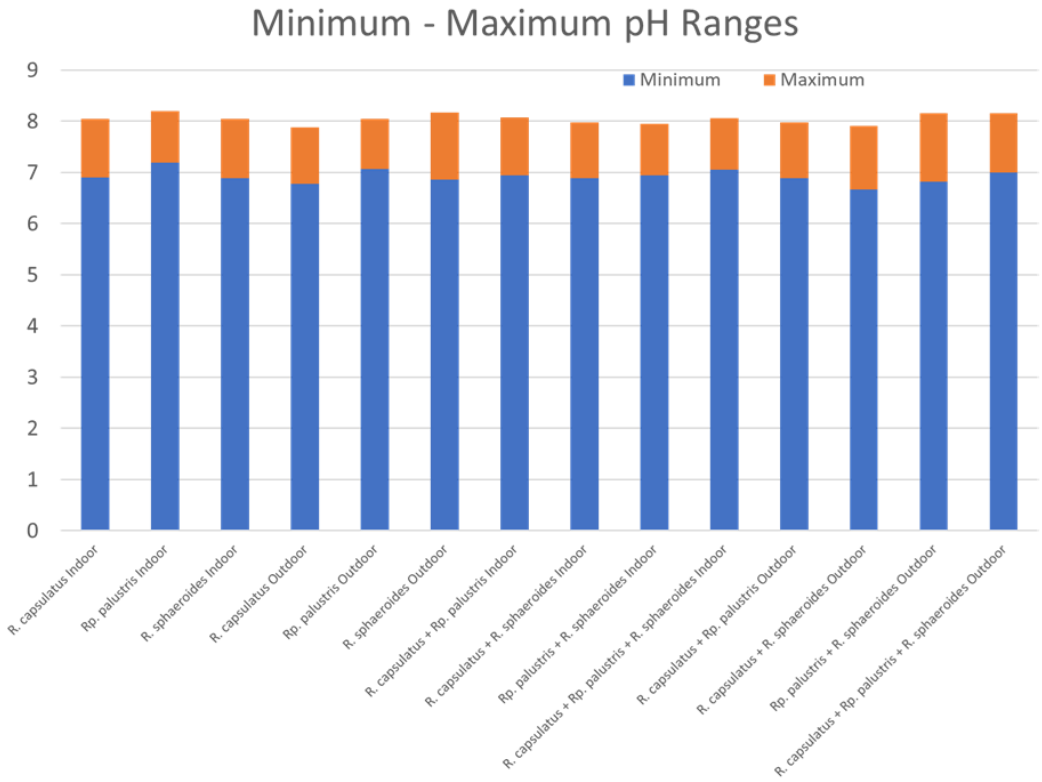

Figure 3.4.8 Minimum and maximum pH ranges for 150 mL single-cultures, double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoor, and under sunlight for outdoor.

Below, the cumulative hydrogen production in both rounds are given for the experimental groups. *R. capsulatus* hup- (YO3) has yielded highest performances.

Co-cultivation has not provided performance advantages. Note that, the unit is given as L/L_C, and the sums of the hydrogen production values for rounds shall not be added to each other and regarded as cumulative hydrogen production performances, since in two rounds, the volume of the culture is doubled with respect to each round.

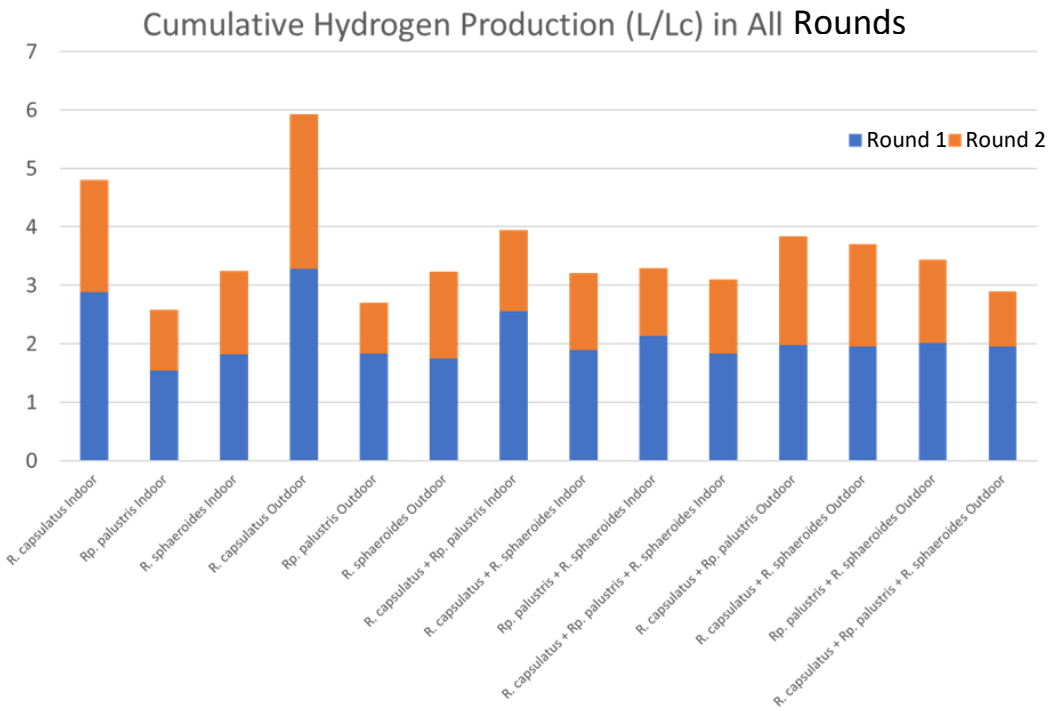

Figure 3.4.9 Cumulative H_2 Production for 150 mL single-cultures, double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux for indoor, and under sunlight for outdoor.

Next, 60 mM acetate concentration was experimented under 4000 lux illuminations to observe the effect of the acetate concentration on immobilized PNSB and

immobilized and co-cultured PNSB.

pH profiles are given in Figure 3.4.10. pH values have increased slower than the previous experimental groups to lower highest pH values, and have fluctuated as expected. However, the final pH values were found comparable to the previous experiments performed indoor with 40 mM acetate under 2500 lux, and outdoor with 40 mM acetate. pH values and fluctuations were within expected range.

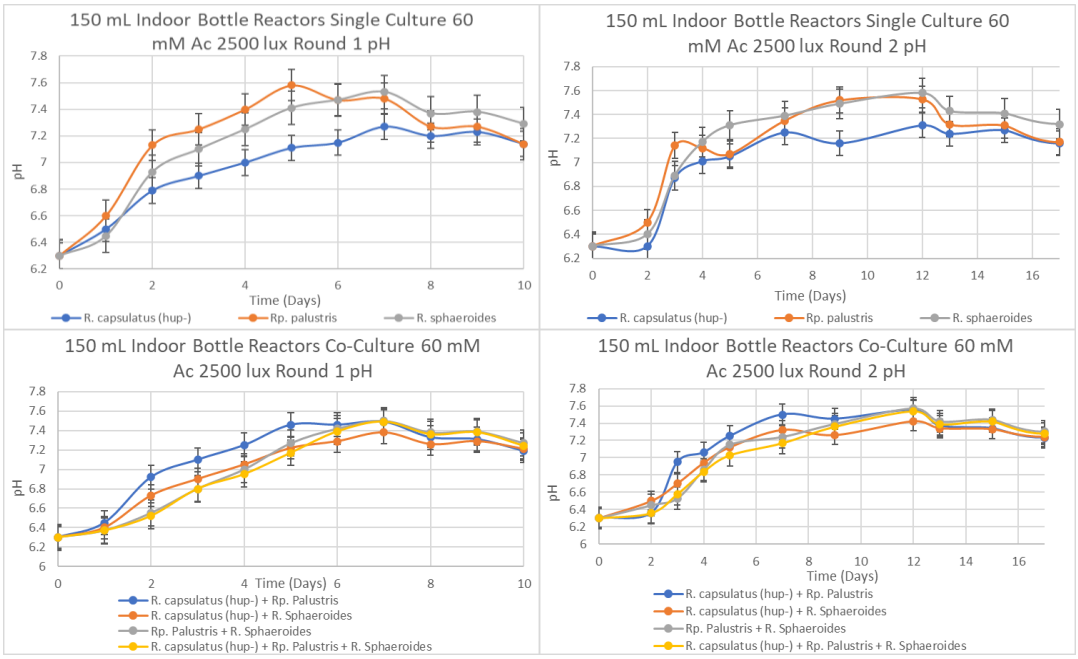

Figure 3.4.10 pH variation for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

Hydrogen production profiles are given in Figure 3.4.11. Hydrogen production performance was relatively low during the first round, but improved during the

second round. However, a significant improvement in general, in comparison with the groups supplemented with 40 mM acetate above was observed. Additionally, both *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) seem to have yielded significantly better results with 60 mM acetate. Especially *Rp. palustris* (DSMZ 127) have yielded comparable performance to *R. capsulatus* hup- (YO3), even higher, during the second round. However, a significant gain over co-cultivation could not be observed.

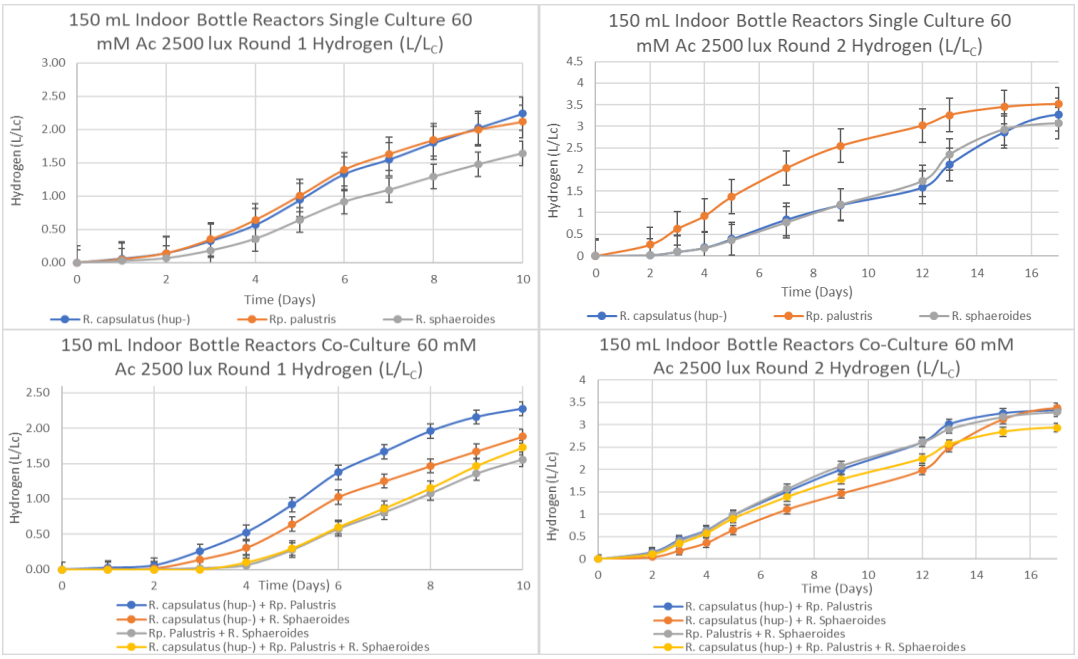

Figure 3.4.11 H_2 Production for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

Acetate utilization is given in Figure 3.4.12. Substrate was again consumed within 10 days, despite higher initial concentration. Although the substrate consumption

was completed, experiment was continued, since hydrogen production has continued. Substrate might have been converted to other metabolites, which might have been consumed on the following days.

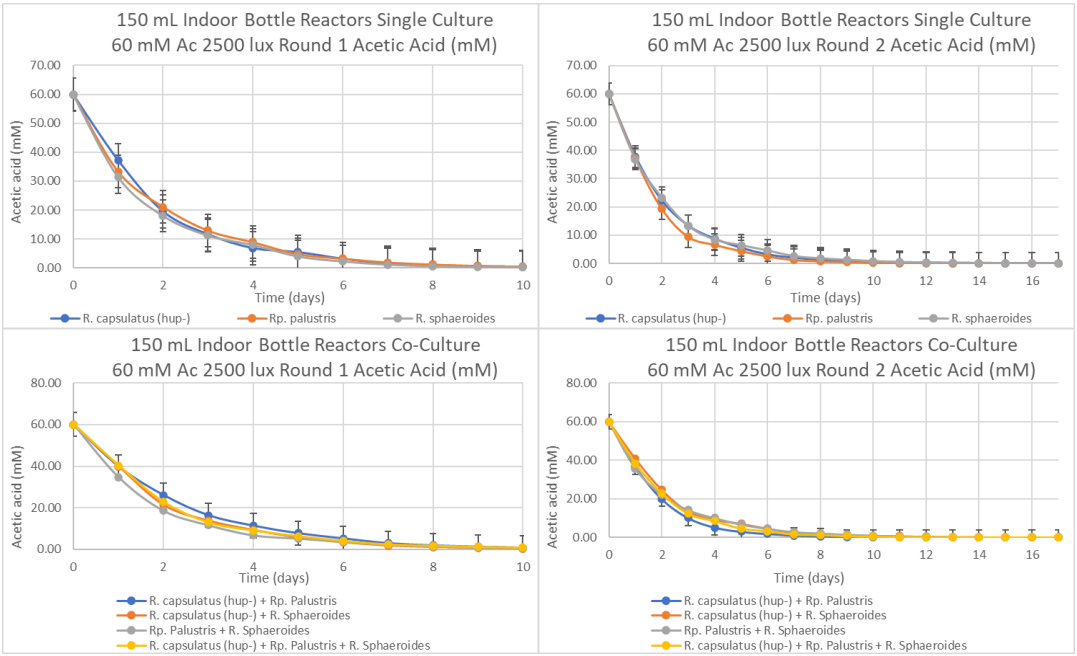

Figure 3.4.12 Acetic acid utilization for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

Next, in order to compare the effect of higher light intensity, experimentations under 4000 lux and 40 mM acetate concentration were performed. Below in the Figure 3.4.13, pH profiles are given. pH ranges, rises and fluctuations were found to be within desirable range.

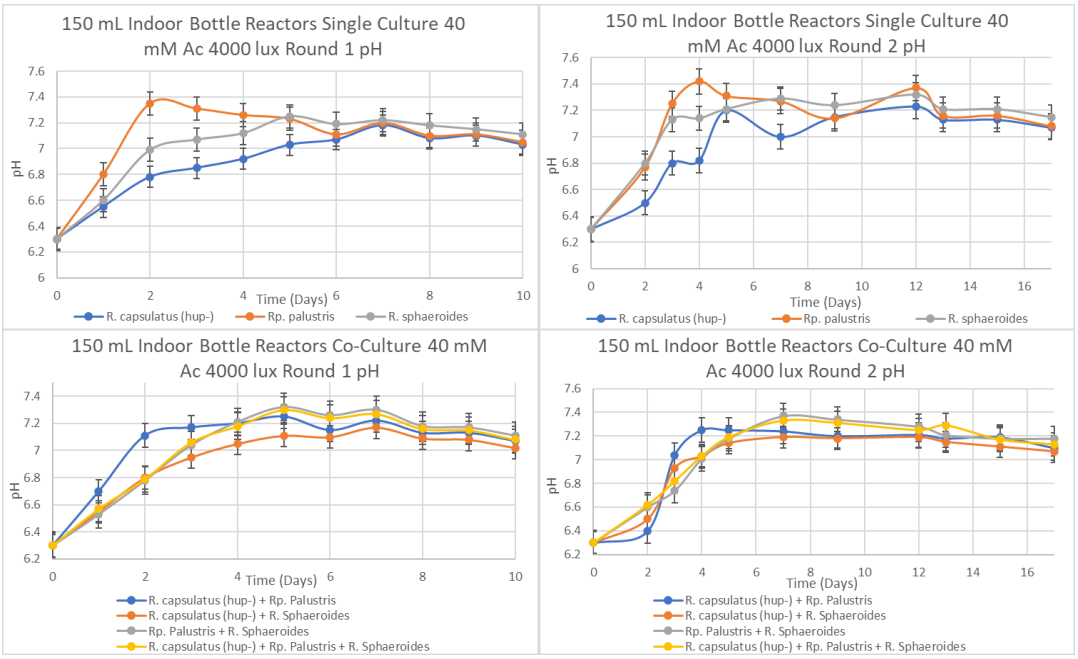

Figure 3.4.13 pH variation for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux.

Hydrogen production profiles are given in Figure 3.4.14. Hydrogen production was found highest for *R. capsulatus* hup- (YO3) single cultures in both rounds. *R. capsulatus* hup- (YO3) + *Rp. palustris* (DSMZ 127) have yielded similar performance during the second round. Also, the hydrogen performances were higher than the 40 mM acetate and 2500 lux groups, and comparable to the 40 mM acetate outdoor groups. Therefore, 4000 lux light intensity over 2500 lux, seem to have improved hydrogen production performance for 40 mM initial acetate concentrations. However, 2500 lux 60 mM acetate groups still yielded higher hydrogen production. Co-cultivation though, was still not observed to confer performance advantages over single cultures.

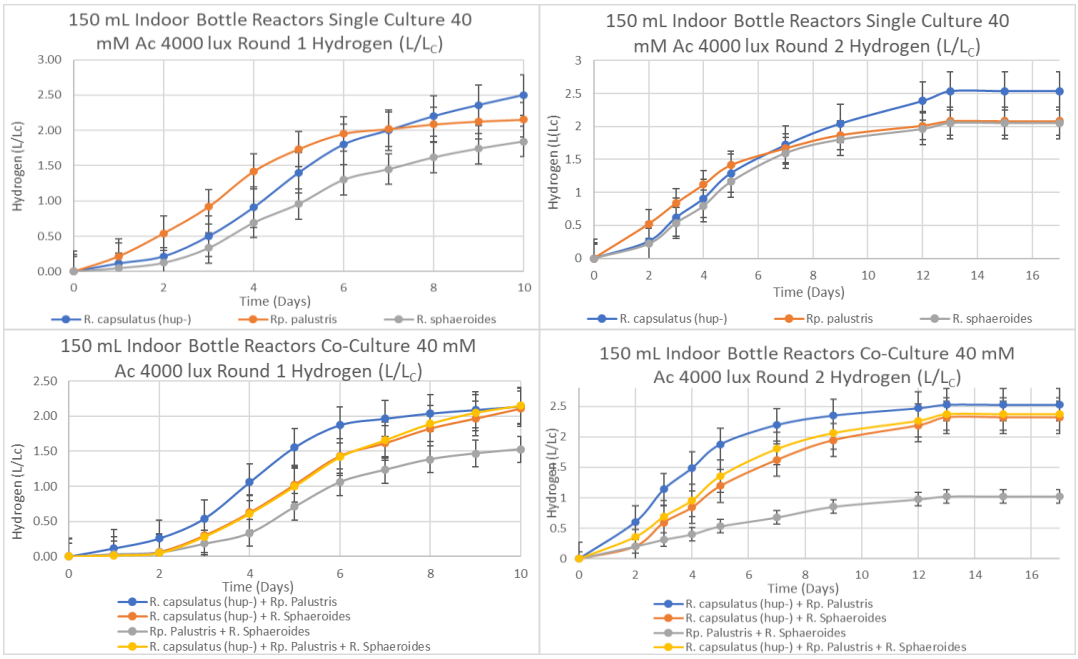

Figure 3.4.14 H_2 Production for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux.

Figure 3.4.15 below shows acetatic acid utilization. Substrate was found to be consumed within 6-9 days. Although the substrate consumption was completed, experiment was continued, since hydrogen production has continued. Substrate might have been converted to other metabolites, which might have been consumed on the following days.

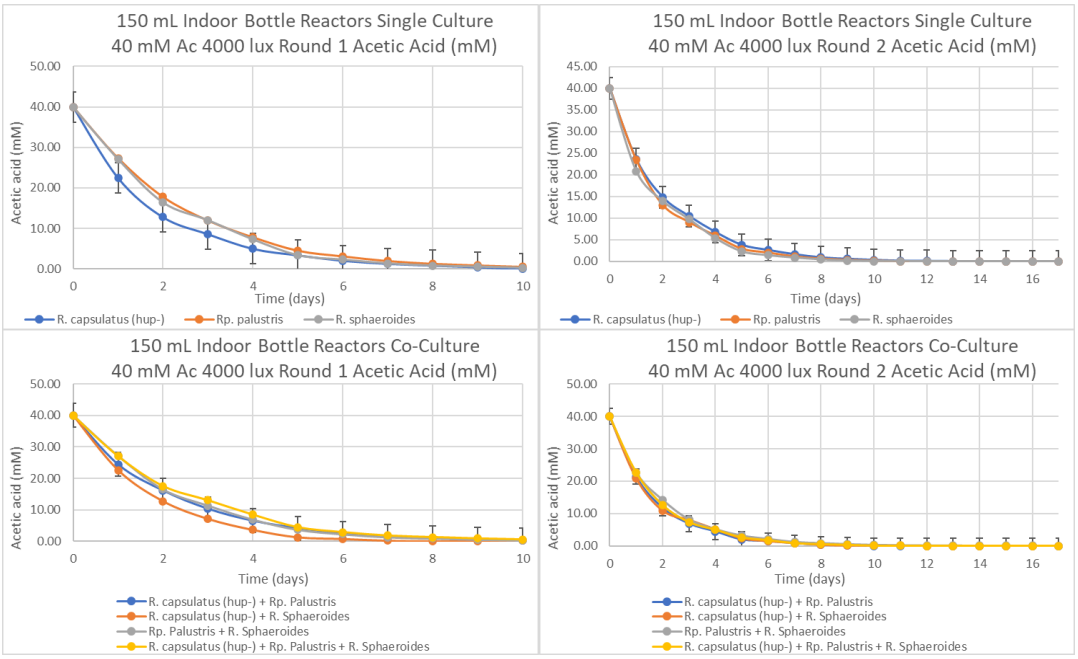

Figure 3.4.15 Acetic acid utilization for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux.

In order to investigate the effects of using both higher acetate concentration and higher light intensity, experiments with 60 mM initial acetate concentrations under 4000 lux light intensity were performed.

Below is the Figure 3.4.16 depicting the pH profile for 60 mM acetate and 4000 lux groups. pH ranges, rises and fluctuations were found to be within desirable range.

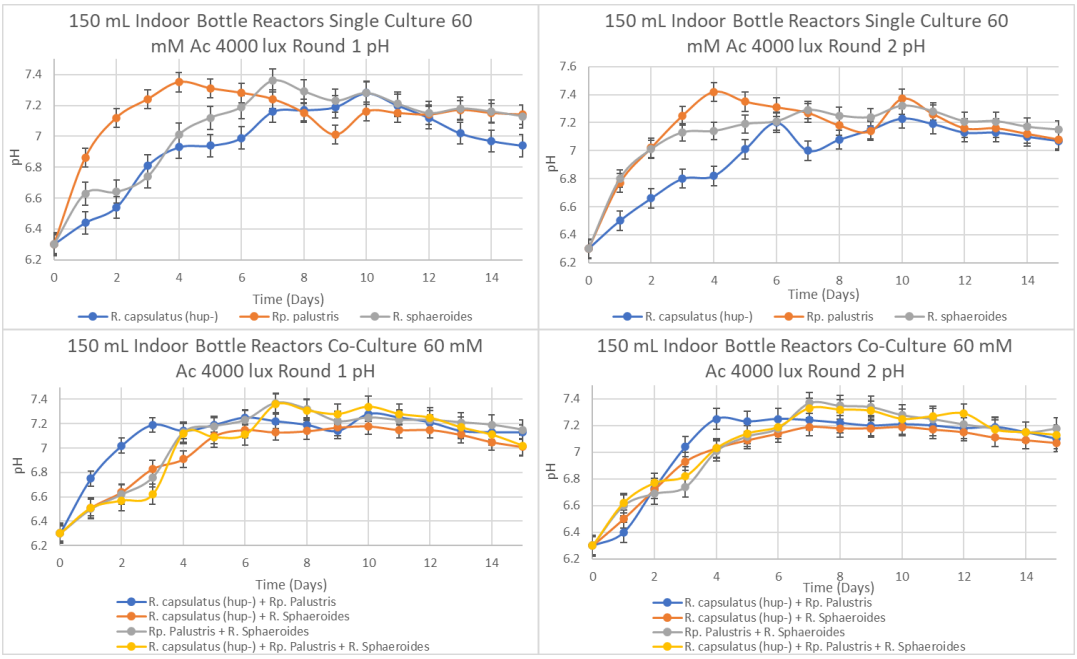

Figure 3.4.16 pH variation for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux.

Figure 3.4.17 depicts the hydrogen production profile for the 60 mM acetate and 4000 lux experiments. Hydrogen production performances were found to be increased against all other previously experimented groups in general. Round 1 have led to higher performances than round 2. Hydrogen production performances of the single cultures within the 60 mM acetate and 4000 lux group was observed comparable with each other among each round. *Rp. palustris* (DSMZ 127) has led to highest hydrogen production during round 1, and meanwhile, the single culture of *R. capsulatus* hup- (YO3) and the triple co-culture have led to lowest hydrogen productivities. On the second round though, the triple co-culture and the single culture of *R. capsulatus* hup- (YO3) have exhibited the second and the third best performances. Hence on 4000 mM acetate and under 4000 lux, different single and

co-cultures were found to exhibit close performances. On the previous trials with different substrate concentrations and light intensities, lower substrate and lower light intensity conditions seemed to cause disadvantages for performing with their full potentials for different single culture and co-culture groups, that is, some of the differences between the performances of the experimental groups have seemed to diminish when the substrate concentration was increased, and the differences of some others have seemed to diminish when the light intensity was increased. This time, when both the substrate concentration and the light intensity was increased, experimental groups have performed even closer to each other. However, immobilization of co-cultures, yet have not exhibited performance advantages on these trials.

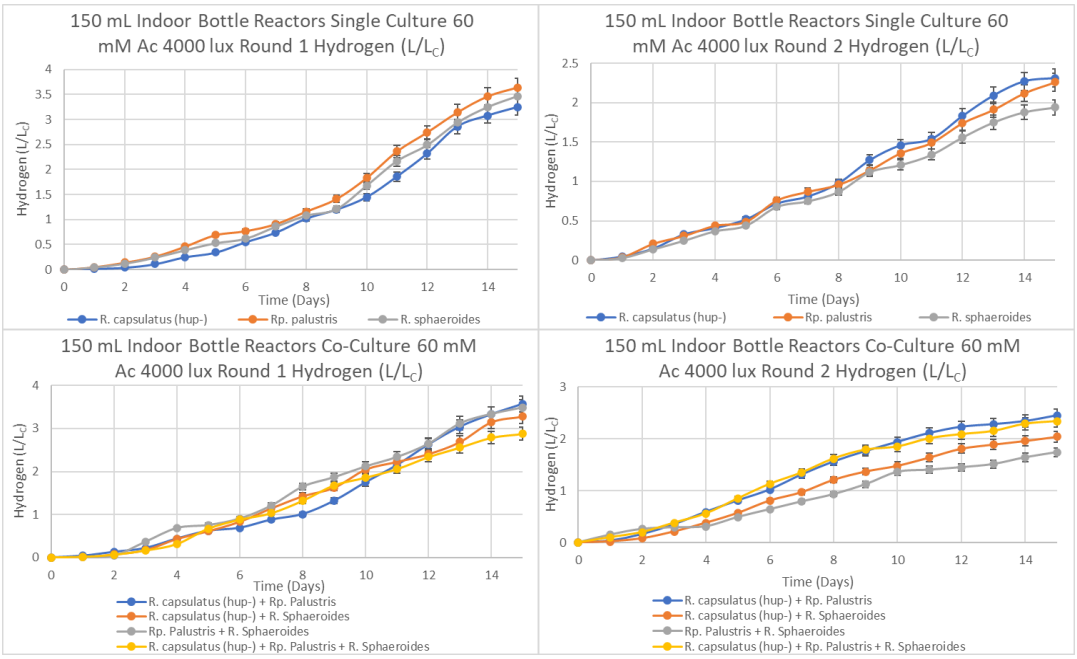

Figure 3.4.17 H_2 Production for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux.

Acetate consumptions are given in Figure 3.4.18. Bacteria have seemed to consumed acetate within 5 to 10 days. Although the substrate consumption was completed, experiment was continued, since hydrogen production has continued. Substrate might have been converted to other metabolites, which might have been consumed on the following days.

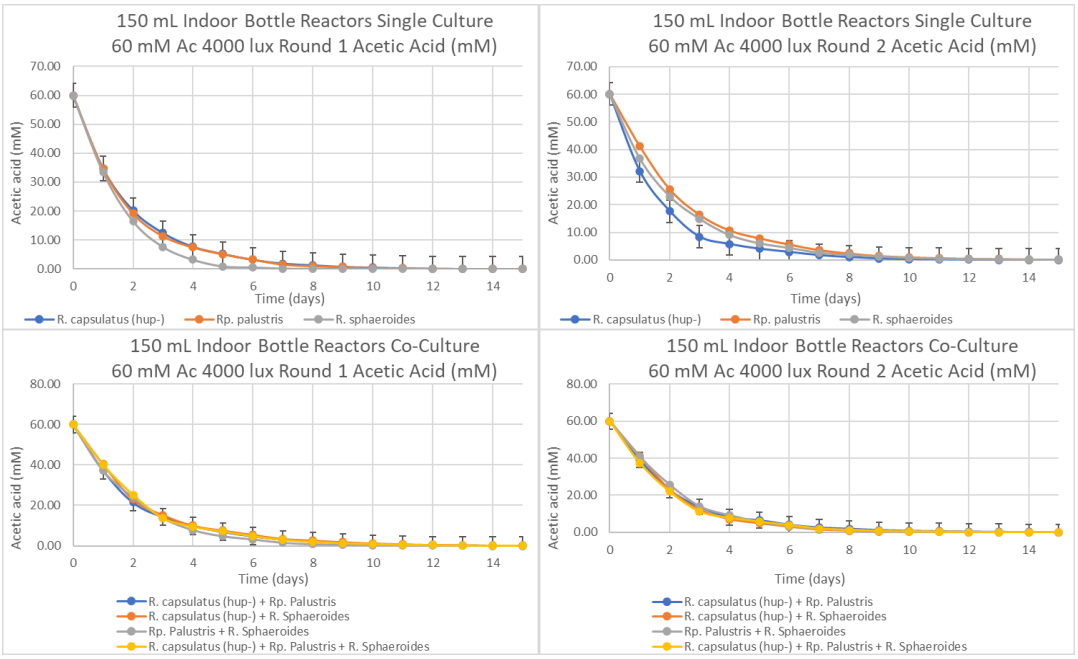

Figure 3.4.18 Acetic acid utilization for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux.

Figure 3.4.19 depicts the minimum and maximum pH values of all experimental groups operated inside 150 mL reactors. pH values were found to be mostly consistent for different single and co-cultures among different rounds with different acetate concentrations and light intensities. However, the 40 mM acetate 2500 lux and 40 mM acetate and outdoor groups have escalated to higher maximum pH values, which was probably due to some experimental errors, as explained above.

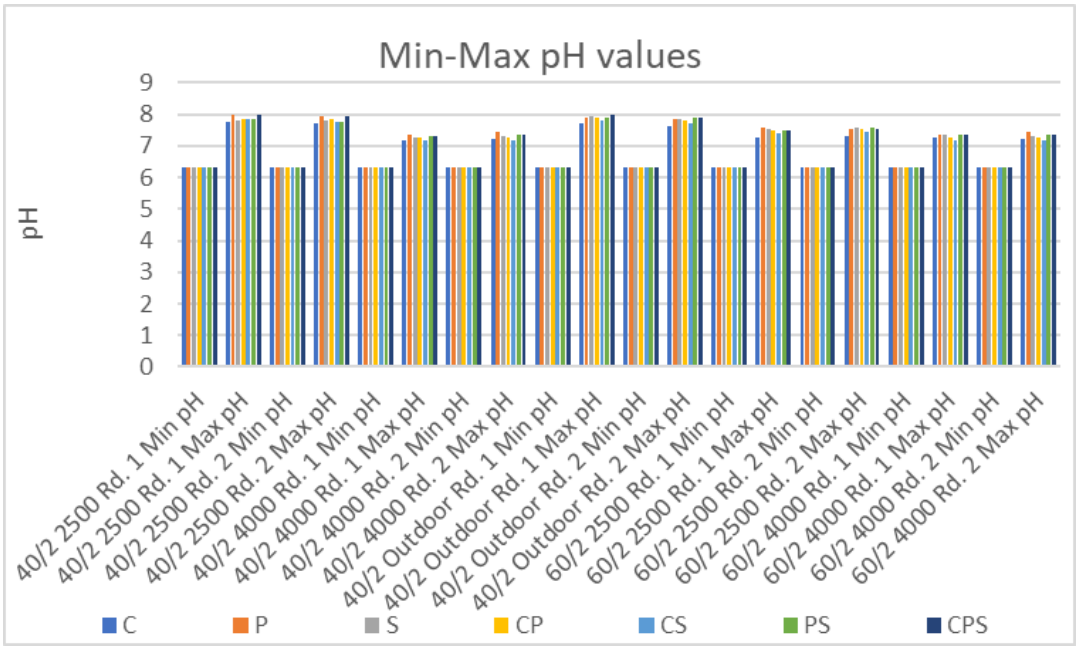

Figure 3.4.19 Minimum and Maximum pH values for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate and 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux and 4000 lux for indoor, and under sunlight for outdoor.

Figures 3.4.20 and 3.4.21 show cumulative hydrogen production for all groups, and Figure 3.4.22 shows hydrogen production for all groups with the individual contributions of separate rounds. As clearly seen, utilization of higher acetate concentrations and higher light intensities has increased hydrogen production generally. Also, if the effects of higher light intensities versus higher acetate concentrations on hydrogen production performances are to be compared, higher light intensities seem to enhance hydrogen production performances at a greater extent than higher acetate concentrations. Higher acetate concentrations alone, may also lead to induction of other metabolic activities, such as production of PHB,

whereas higher light intensities may shift the metabolism to primarily hydrogen production. Additionally, from those graphs, information regarding the performances of individual single and co-culture groups may be deduced. In general, *R. capsulatus* hup- (YO3) have exhibited the best performance among single and co-cultures. Co-cultivation of immobilized PNSB have not yielded substantial hydrogen production performance gains, mostly resulting in values between the performances of the single cultures of the constituent strains. For single cultures, the performances of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) have followed *R. capsulatus* hup- (YO3). For Co-cultures, the di-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) have performed the best, followed by the di-culture of *R. capsulatus* hup- (YO3) and *R. sphaeroides* O.U.001 (DSMZ 5864) and the di-culture of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864). The tri-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) have led to generally poorest performing hydrogen production activities, which also does not comply with the previous co-cultivation on suspended media study (Baysal, 2012).

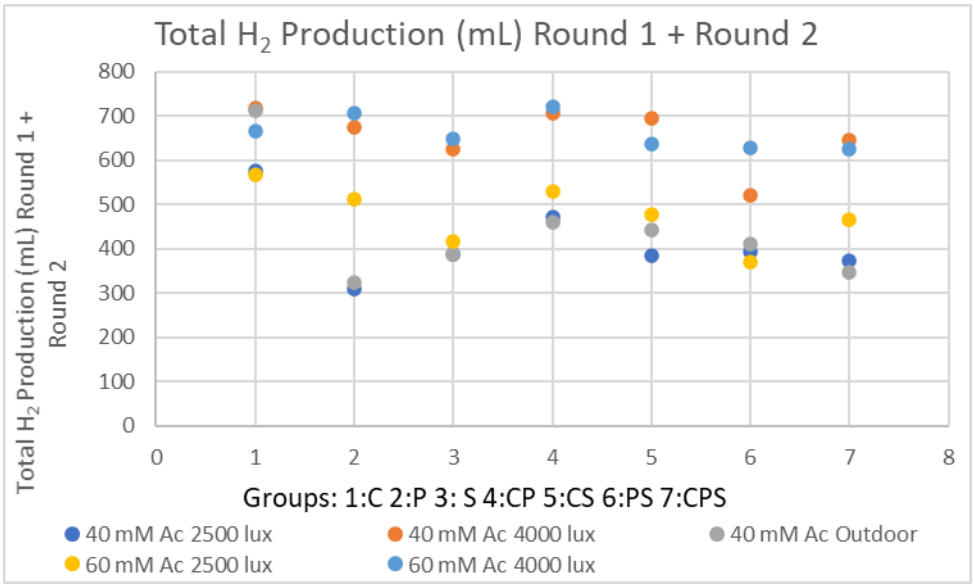

Figure 3.4.20 Dot graph for the Total H_2 Production in rounds 1 and 2 for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate and 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux and 4000 lux for indoor, and under sunlight for outdoor.

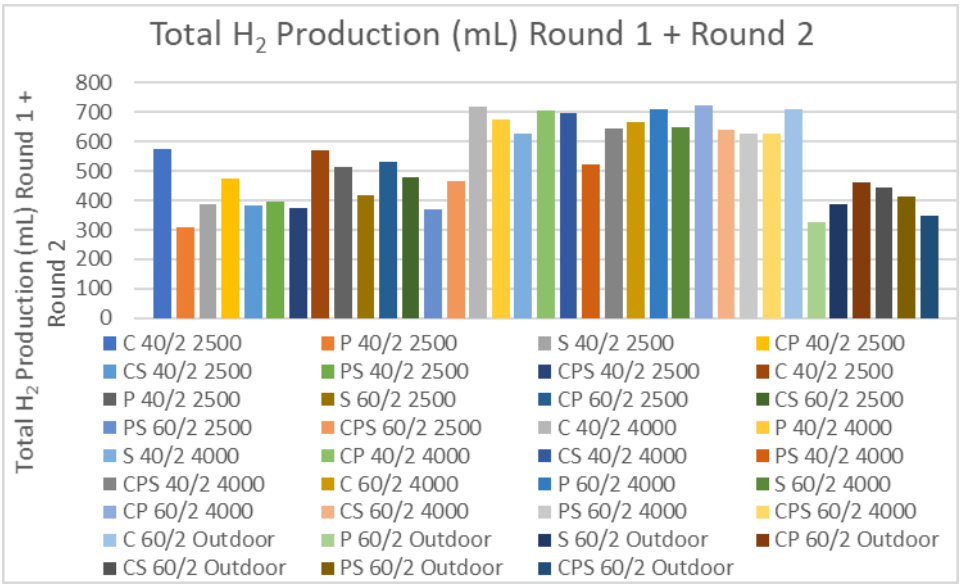

Figure 3.4.21 Bar graph for the Total H_2 Production in rounds 1 and 2 for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate and 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux and 4000 lux for indoor, and under sunlight for outdoor.

Figure 3.4.22 Total H_2 Production in round 1 and round 1 + round 2 for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate and 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux and 4000 lux for indoor, and under sunlight for outdoor.

Figure 3.4.23 depicts hydrogen production performances of each single and co-culture of each strain for each round of each acetate concentration and light intensity group. Bars on the top show the results of the triple-co-cultures. The co-culture of

each strain with both others is the triple co-culture of the three cultures. The co-culture of each strain with itself is its single culture. As seen from the figure, single culture of *R. capsulatus* hup- (YO3) have yielded the highest hydrogen production performance on all rounds of 40 mM acetate 2500 lux, 40 mM acetate outdoors and 40 mM acetate 4000 lux. *Rp. palustris* (DSMZ 127) have yielded the highest hydrogen production performance on 60 mM acetate 2500 lux Round 2 and 60 mM acetate 4000 lux Round 1. On the other hand, on 60 mM acetate 2500 lux Round 1 and 60 mM acetate 4000 lux Round 2, di-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) have yielded the highest hydrogen production performance. On 40 mM acetate 4000 lux Round 2, hydrogen production performance of the di-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) was marginally higher than the hydrogen production performance of the single-culture of *R. capsulatus* hup- (YO3). Tri-cultures have only yielded relatively comparable results to best performing single cultures under 4000 lux. Whereas the di-culture of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) have yielded relatively lowest hydrogen production on 40 mM acetate 4000 lux experiments. *R. capsulatus* hup- (YO3) has yielded its relatively lowest hydrogen production performances on 60 mM acetate. *Rp. palustris* (DSMZ 127), on the other hand, has yielded it relatively highest hydrogen production performances on 60 mM acetate, as its single culture, or as the di-culture with *R. capsulatus* hup- (YO3). Hence *R. capsulatus* hup- (YO3) seem to dominate hydrogen production under low acetate concentrations, and *Rp. palustris* (DSMZ 127) seem to dominate hydrogen production under high acetate concentrations. Therefore, although, co-cultivation has not conferred significant performance gains on immobilized cultures, such as suspended cultures, actually the immobilized di-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) might be the optimum co-cultured PNSB couple, which may yield high hydrogen production under various acetate concentrations and light intensities, if not the highest.

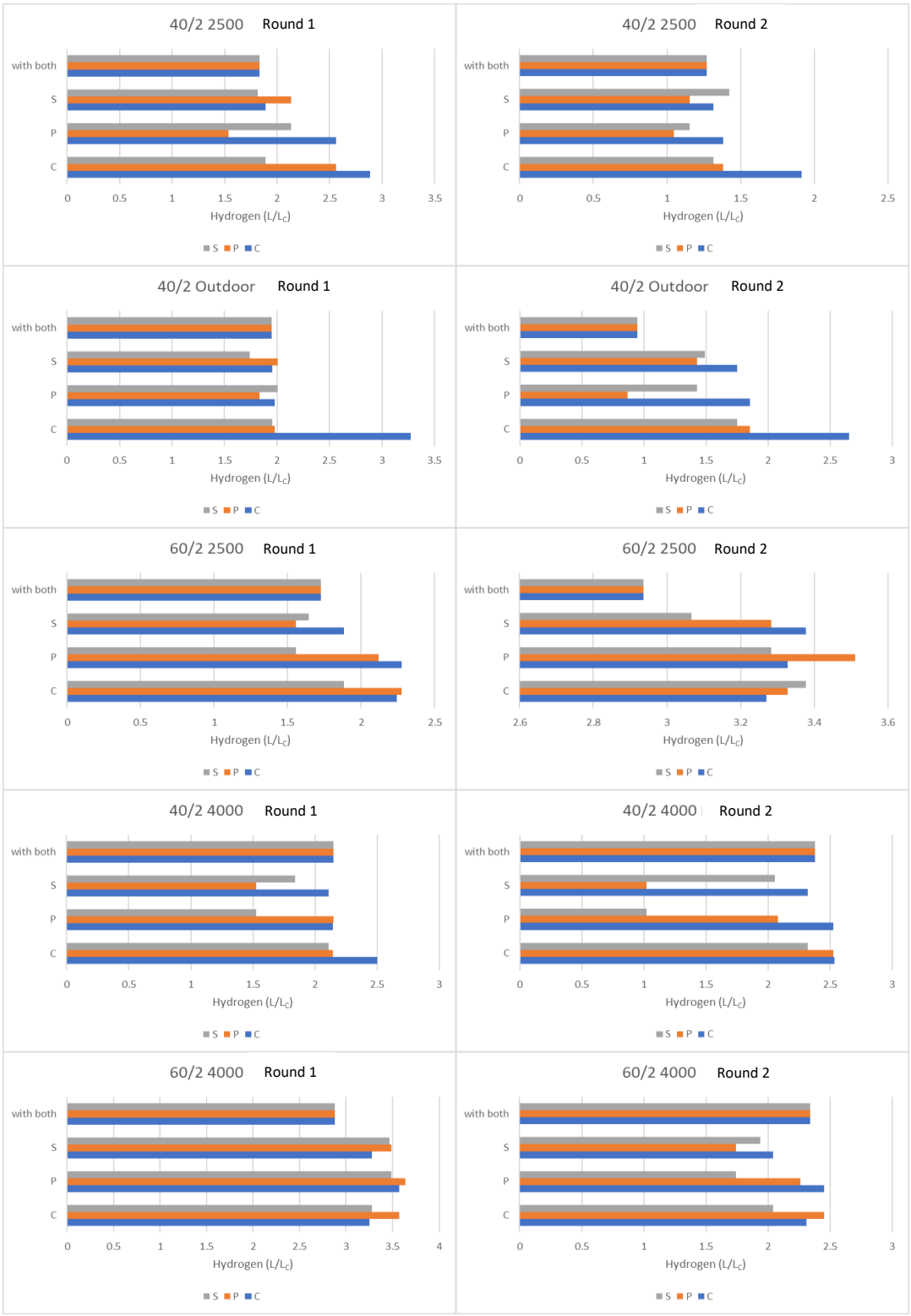

Figure 3.4.23 Total H_2 Production of 150 mL single cultures and double and triple co-cultures of each strain among *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ

127) and *R. sphaeroides* O.U.001 (DSMZ 5864) with each other one and with both of the other ones, in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate and 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux and 4000 lux for indoor, and under sunlight for outdoor.

Table 3.4.9 lists the productivity values for PNSB. The highest productivities were performed by 60 mM acetate 4000 lux round 1 group. Their average is 0.417 mmol H_2/L.h. The second-best performing group was, 40 mM acetate 4000 lux round 1 group. Their average is 0.383 mmol H_2/L.h. The highest performing culture was, the single culture *R. capsulatus* hup- (YO3) with 0.3432 H_2/L.h average. It was followed by the co-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) with 0.3017 H_2/L.h average. Hence, higher light intensity groups seem to lead to higher productivities.

Table 3.4.9 Hydrogen Productivity (mmol H_2/L.h) of single-cultures and co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864).

Productivity	40/2 2500		40/2 4000		40/2 Outdoor		60/2 2500		60/2 4000	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
C	0.358	0.174	0.465	0.363	0.406	0.202	0.417	0.358	0.403	0.286
P	0.143	0.045	0.400	0.298	0.171	0.051	0.394	0.384	0.451	0.280
S	0.225	0.160	0.343	0.294	0.162	0.073	0.305	0.336	0.430	0.241
CP	0.238	0.124	0.398	0.362	0.245	0.116	0.423	0.364	0.443	0.304
CS	0.176	0.111	0.392	0.332	0.242	0.154	0.350	0.370	0.407	0.253
PS	0.198	0.115	0.284	0.146	0.249	0.111	0.290	0.359	0.433	0.216
CPS	0.170	0.082	0.400	0.340	0.241	0.088	0.321	0.321	0.357	0.290

Tables 3.4.10, 3.4.11 and 3.4.12 list the hydrogen yield, substrate conversion efficiency and hydrogen production values for PNSB. The highest yields were performed by 60 mM acetate 4000 lux round 1 group. Their average is 2.506 mol H_2/mol Acetate, 62.659% and 3.368 L/L_C. The second-best performing group was, 60 mM acetate 2500 lux round 2 group. Their average is 2.420 mmol H_2/L.h and 60.513% 3.331 L/L_C. The highest performing culture was, the single culture *R. capsulatus* hup- (YO3) with 2.523 mol H_2/mol Acetate and 64.453% 2.650 L/L_C average. It was followed by the co-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) with 2.179 mol H_2/mol Acetate and 56.202% 2.334 L/L_C average. Hence, higher substrate concentration groups seem to lead to hydrogen yields and substrate conversion efficiencies.

Table 3.4.10 Hydrogen Yield (mol H_2/mol Acetate) of single-cultures and co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864).

Yield	40/2 2500		40/2 4000		40/2 Outdoor		60/2 2500		60/2 4000	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
C	3.218	2.086	2.790	2.830	3.655	2.421	1.668	2.433	2.418	1.719
P	1.718	0.403	2.400	2.322	2.046	0.617	1.575	2.612	2.708	1.682
S	2.024	1.438	2.055	2.291	1.944	0.880	1.221	2.282	2.582	1.443
CP	2.858	1.485	2.390	2.821	2.207	1.389	1.693	2.476	2.656	1.823
CS	2.111	1.330	2.353	2.592	2.179	1.851	1.401	2.513	2.440	1.518
PS	2.381	1.383	1.702	1.141	2.241	1.327	1.159	2.443	2.597	1.295
CPS	2.043	0.989	2.400	2.654	2.173	1.082	1.283	2.185	2.143	1.741

Table 3.4.11 Substrate Conversion Efficiency (%) of single-cultures and co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864).

SCE	40/2 2500		40/2 4000		40/2 Outdoor		60/2 2500		60/2 4000	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
C	80.450	52.161	69.754	70.762	91.378	74.095	41.698	60.816	60.454	42.969
P	42.938	10.076	59.989	58.051	51.153	24.182	39.373	65.311	67.708	42.039
S	50.611	35.962	51.386	57.276	48.596	41.543	30.537	57.044	64.546	36.086
CP	71.460	37.125	59.756	70.530	55.184	51.773	42.318	61.901	66.406	45.573
CS	52.781	33.250	58.826	64.794	54.486	48.828	35.032	62.831	61.012	37.946
PS	59.524	34.567	42.550	28.522	56.036	39.760	28.987	61.074	64.918	32.366
CPS	51.076	24.724	59.989	66.344	54.331	26.429	32.087	54.615	53.571	43.527

Table 3.4.12 Hydrogen Production (L/L_C) values of single-cultures and co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864).

Total H_2	40/2 2500		40/2 4000		40/2 Outdoor		60/2 2500		60/2 4000	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
C	2.88	1.87	2.50	2.54	3.28	2.17	2.24	3.46	3.25	2.31
P	1.54	0.36	2.15	2.08	1.83	0.55	2.12	3.54	3.64	2.26
S	1.81	1.29	1.84	2.05	1.74	0.79	1.64	3.16	3.47	1.94
CP	2.56	1.33	2.14	2.53	1.98	1.24	2.28	3.36	3.57	2.45
CS	1.89	1.19	2.11	2.32	1.95	1.66	1.88	3.46	3.28	2.04
PS	2.13	1.24	1.53	1.02	2.01	1.19	1.56	3.33	3.49	1.74
CPS	1.83	0.89	2.15	2.38	1.95	0.97	1.73	3.01	2.88	2.34

Table 3.4.13 list the final pH values for PNSB. All results are within the expected range and close to each other.

Table 3.4.13 Final pH Values of single-cultures and co-cultures of Immobilized PNS Bacteria, experimented in 150 mL reactors. C: *R. capsulatus* hup- (YO3) P: *Rp. palustris* (DSMZ 127) S: *R. sphaeroides* O.U.001 (DSMZ 5864).

pH	40/2 2500		40/2 4000		40/2 Outdoor		60/2 2500		60/2 4000	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
C	6.94	6.86	7.03	7.07	6.87	6.69	7.14	7.16	6.94	7.07
P	7.34	7.16	7.05	7.08	7.23	7.05	7.14	7.17	7.14	7.08
S	7.00	6.83	7.11	7.15	6.98	6.82	7.29	7.32	7.13	7.15
CP	7.05	6.91	7.07	7.10	6.97	6.96	7.19	7.23	7.13	7.10
CS	7.03	6.89	7.02	7.07	6.80	6.63	7.21	7.24	7.01	7.07
PS	7.02	6.85	7.11	7.18	6.91	6.75	7.27	7.30	7.15	7.18
CPS	7.16	6.97	7.08	7.13	7.10	6.93	7.24	7.28	7.02	7.13

3.5 Hydrogen Production by Immobilized Single Cultures of PNSB

Experiments with 1.4 L reactors were performed as 1 run and 1 round. Experiments with 300 mL reactors were performed as 2 runs, the first run having 2 rounds and the second run having 5 rounds. Experiments with 150 mL reactors were performed as 1 run and 2 rounds. The total hydrogen production occurred the highest among experimented groups for single culture *Rp. palustris* (DSMZ 127) with 3.60 (L/Lc) during the first run of the experiments performed with the 150 mL reactors using 60 mM acetate concentration and 4000 lux light intensity. Apart from this, in general, the total hydrogen production occurred the highest on average for single culture *Rp. palustris* (DSMZ 127) during the experiments performed with the 150 mL reactors using 60 mM acetate concentration and either 2500 lux or 4000 lux light intensity. However, the total hydrogen production occurred the highest on average for single culture *R. capsulatus* (YO3) during the experiments performed with the 150 mL reactors using 40 mM acetate concentration. Therefore, highest hydrogen production performances were observed for the single cultures of PNSB, namely the single cultures of *Rp. palustris* (DSMZ 127) for the higher acetate concentration experimented (60 mM) and the single cultures of *R. capsulatus* (YO3) for the lower acetate concentration experimented (40 mM) throughout this study. These results do not comply with the suspended culture experiments, which have shown that, co-cultivation of PNSB might have an advantageous synergistic effect on hydrogen production (Baysal, 2012).

3.6 Hydrogen Production by Immobilized Different Co-Cultures of PNSB

Experiments with 1.4 L reactors were performed as 1 run and 1 round. Experiments with 300 mL reactors were performed as 2 runs, the first run having 2 rounds and the second run having 5 rounds. Experiments with 150 mL reactors were performed as 1 run and 2 rounds. Although, as stated above, on average, the total hydrogen production was recorded as the highest for outdoors and indoors with the single cultures of *R. capsulatus* (YO3) and *R. sphaeroides*, the di-culture of *R. capsulatus* (YO3) and *R. sphaeroides* have yielded the highest hydrogen production performances on the first round of the experiments with 60 mM acetate concentration and 2500 lux light intensity, and on the second round of the experiments with 60 mM acetate concentration and 4000 lux light intensity. The tri-cultures, have only yielded hydrogen production performances comparable to the single cultures under 4000 lux light intensity. These results do not comply with the suspended culture studies, during which, the triple cultures have conferred highest hydrogen production, followed by the di-cultures and the single cultures (Baysal, 2012). Still, the results revealed higher yields than the previous trials with suspended cultures (1.48 to 1.92 L/L_c) and the ones reported by Elkahlout *et al.,* (1.5 to 1.85 L/L_c) involving immobilized cultures. Although a distinctive synergy might not be stipulated when the hydrogen production performances of the co-cultured PNSB are examined, actually, *R. capsulatus* hup- (YO3) was found to be advantageous under lower acetate concentrations, and *Rp. palustris* (DSMZ 127) was found to be advantageous under higher acetate concentrations. Additionally, their di-cultures were found to exhibit comparable hydrogen production performances, if not higher. Hence although a synergistic effect of co-cultivating PNSB on hydrogen production might not be suggested, the di-culture of *R. capsulatus* hup- (YO3) and *Rp. palustris* (DSMZ 127) might provide advantages, as this di-culture may provide comparably high hydrogen production under low acetate concentrations such as the single culture

of *R. capsulatus* hup- (YO3) and comparably high hydrogen production under high acetate concentrations such as the single culture of *Rp. palustris* (DSMZ 127).

3.7 pH Changes of Single Cultures and Double and Triple Co-Cultures of Immobilized PNSB During Hydrogen Production

Throughout the experiments, the pH values obtained were found to be within expected and normal range. However, for the 40 mM acetate concentration and 2500 lux indoor illumination and outdoor illumination groups, although the cultures were prepared at pH 6.3, a pH increase to 7.4-8.0 was observed on the first day of the experiments. pH tended to increase in the following 2-3 days, then showed a tendency to decrease. Even though bacterial stress was minimized by keeping the bacteria at a suitable temperature for more prolonged periods before being poured, still the bacteria was experiencing stress, even though minimized, which might be responsible for this initial abnormal pH rise. At the end of the experiments, pH values decreased to 6.6-7.2 intervals.

3.8 Substrate Utilization by Single Cultures and Double and Triple Co-Cultures of Immobilized PNSB During Hydrogen Production

HPLC data revealed that the acetic acid content of the medium was significantly metabolized by all experimental groups during the first 5 days and almost completely metabolized on the 10^{th} day. However, as seen on the data for the 15^{th} and 20^{th} days, although the remaining acetic acid concentrations were minimal, they exhibited fluctuations over time. These results comply with previous studies and may imply

the dynamic nature of the PNSB organic acid metabolism, leading to both consumption and synthesis (Savasturk *et al.,* 2018, Oflaz *et al.,* 2021).

3.9 Comparison of Hydrogen Production Performances of Immobilized Single Cultures and Co-Cultures of PNSB with Literature and Suspension Cultures

Table 3.9.1 compares the hydrogen production performances obtained in this study with another immobilization study in the literature (Elkahlout *et al.,* 2019). Here, best performing experimental samples are compared. Total hydrogen production and hydrogen yield performances were found to be in between the other studies, productivity performances were observed to be lower, and substrate conversion efficiencies were observed to be higher than the other studies.

Table 3.9.1 Total Hydrogen Produced (L/L_C), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h) and Substrate Consumption (%) comparison of Immobilized PNS Bacteria from acetate with another study regarding Hydrogen Production by immobilized PNS Bacteria (Elkahlout *et al.*, 2019). Comparison of the best performing experimental samples. TH_2: Total Hydrogen Production, H_2Y: Hydrogen Yield, H_2P: Hydrogen Productivity, SCE: Substrate Conversion Efficiency.

	60 mM Acetate Elkahlout *et al.,* 2019)	150 ml 40/2 Outdoor Round 1 *R. capsulatus*	150 mL 60/2 4000 lux Round 1 *Rp. palustris*
TH_2	5.2	3.280	3.640
H_2Y	3.53	3.655	2.708
H_2P	1.3	0.406	0.451
SCE		91.378	67.708

Table 3.9.2 compares the hydrogen production performances obtained in this study with the previous suspended culture study (Baysal, 2012). Here, best performing experimental samples are compared. Total hydrogen production, hydrogen yield and substrate conversion efficiencies were observed to be higher, whereas productivity performances were observed to be lower than the suspended culture study.

Table 3.9.2 Total Hydrogen Produced (L/L_C), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h) and Substrate Consumption (%) comparison of Immobilized PNS Bacteria from acetate with the previous study regarding Hydrogen Production by suspended PNS Bacteria (Baysal, 2012). Comparison of the best performing experimental samples. TH_2: Total Hydrogen Production, H_2Y: Hydrogen Yield, H_2P: Hydrogen Productivity, SCE: Substrate Conversion Efficiency.

	Suspension		Immobilized	
	CPS (Baysal, 2012)	PS (Baysal, 2012)	150 ml 40 mM Acetate Outdoor Round 1 *R. capsulatus*	150 mL 60 mM Acetate 4000 lux Round 1 *Rp. palustris*
TH_2	1.82	1.77	3.280	3.640
H_2Y	2.61	2.23	3.655	2.708
H_2P	0.90	0.94	0.406	0.451
SCE	65.28	55.64	91.378	67.708

Table 3.9.3 compares the hydrogen production performances obtained in this study with the previous suspended culture study (Baysal, 2012). Here, means of the best performing experimental groups are compared. Total hydrogen production, hydrogen yield and substrate conversion efficiencies were observed to be higher, whereas productivity performances were observed to be lower than the suspended culture study.

Table 3.9.3 Total Hydrogen Produced (L/L_C), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h) and Substrate Consumption (%) comparison of Immobilized PNS Bacteria from acetate with the previous study regarding Hydrogen Production by suspended PNS Bacteria (Baysal, 2012). Comparison of the means of the best performing experimental groups, means of all single and co-cultures within the groups. TH_2: Total Hydrogen Production, H_2Y: Hydrogen Yield, H_2P: Hydrogen Productivity, SCE: Substrate Conversion Efficiency.

	Suspension	Immobilized		
	Mean of 40 mM Acetate (Baysal, 2012)	Mean of 150 mL 60 mM Acetate 4000 lux Round 1	150 mL 40 mM Acetate 4000 lux	150 mL 60 mM Acetate 4000 lux
TH_2	1.587	3.368	2.095	2.761
H_2Y	1.990	2.506	2.338	2.054
H_2P	0.615	0.417	0.344	0.342
SCE	49.495	62.659	58.466	51.365

3.10 Data of All Experiments Performed

Below, Total Hydrogen Production (L/Lc), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h), Substrate Conversion Efficiency (%) and pH data for all single-cultures, and double and triple co-cultures of Immobilized PNS Bacteria are given in Table 3.10.1.

Table 3.10.1 Total Hydrogen Production (L/Lc), Hydrogen Yield (mol H_2/mol Acetate), Hydrogen Productivity (mmol H_2/L.h), Substrate Conversion Efficiency (%) and pH comparison single-cultures, and double and triple co-cultures of Immobilized PNS Bacteria. C: *R. capsulatus* hup- (YO3), P: *Rp. palustris* (DSMZ 127), S: *R. sphaeroides* O.U.001 (DSMZ 5864), I: Indoor, O: Outdoor B: Bi-layer, TH_2: Total Hydrogen Production, H_2Y: Hydrogen Yield, H_2P: Hydrogen Productivity, SCE: Substrate Conversion Efficiency NA: Not Available.

	1.4 L				300 mL Run 1 Round 1								300 mL Run 1 Round 2							
	PO	SO	PSO	BPSO	PI	SI	PSI	BPSI	PO	SO	PSO	BPSO	PI	SI	PSI	BPSI	PO	SO	PSO	BPSO
TH_2	0.35	0.389	0.222	0.395	0.456	0.64	0.564	0.372	0.52	0.128	0.432	0.44	0.165	0.154	0.019	0.044	0.15	0.104	0.196	0.125
H_2P	0.027	0.03	0.017	0.03	0.141	0.198	0.175	0.115	0.161	0.04	0.134	0.136	0.044	0.041	0.005	0.012	0.04	0.028	0.052	0.033
H_2PY	0.39	0.434	0.248	0.441	0.509	0.714	0.629	0.415	0.58	0.143	0.482	0.491	0.184	0.171	0.021	0.049	0.167	0.116	0.219	0.139
SCE	9.765	10.856	6.214	11.033	12.723	17.857	15.737	10.379	14.509	3.571	12.054	12.277	4.607	4.286	0.536	1.232	4.179	2.893	5.464	3.482
pH	6.35	6.37	6.46	6.42	NA	NA	NA	NA	NA	NA	NA	NA	6.78	7.12	6.59	6.51	6.5	6.57	6.54	6.54

Table 3.10.1 continued.

300 mL Run 2 Round 1									300 mL Run 2 Round 2							
	PI	SI	PSI	BPSI	PO	SO	PSO	BPSO	PI	SI	PSI	BPSI	PO	SO	PSO	BPSO
TH_2	1.38	0.588	0.496	0.916	0.697	0.309	0.68	0.266	1.527	1.055	1.185	1.268	1.018	0.707	0.838	0.897
H_2P	0.642	0.273	0.231	0.426	0.324	0.143	0.316	0.124	0.71	0.491	0.551	0.59	0.473	0.329	0.39	0.417
H_2Y	1.54	0.656	0.553	1.022	0.778	0.344	0.759	0.297	1.704	1.177	1.322	1.415	1.136	0.789	0.935	1.001
SCE	38.493	16.397	13.836	25.547	19.447	8.609	18.986	7.414	42.597	29.434	33.061	35.382	28.406	19.728	23.384	25.033
pH	7.54	7.38	7.14	7.29	8.3	7.78	7.29	7.92	7.51	7.35	7.15	7.3	8.22	7.79	7.3	7.56
300 mL Run 2 Round 3									300 mL Run 2 Round 4							
	PI	SI	PSI	BPSI	PO	SO	PSO	BPSO	PI	SI	PSI	BPSI	PO	SO	PSO	BPSO
TH_2	1.585	1.073	1.32	1.407	1.185	0.938	0.961	1.066	1.58	1.079	1.329	1.428	1.184	0.946	0.979	1.069
H_2P	0.737	0.499	0.614	0.654	0.551	0.436	0.447	0.496	0.735	0.502	0.618	0.664	0.55	0.44	0.455	0.497
H_2Y	1.769	1.198	1.473	1.57	1.323	1.047	1.072	1.189	1.764	1.204	1.484	1.594	1.321	1.056	1.092	1.193
SCE	44.233	29.94	36.829	39.246	33.074	26.178	26.802	29.736	44.095	30.098	37.089	39.84	33.023	26.39	27.302	29.833
pH	7.51	7.33	7.14	7.32	8.28	7.78	7.26	7.9	7.31	7.22	6.83	7.12	8.28	7.8	7.24	7.92

Table 3.10.1 continued.

	300 mL Run 2 Round 5								150 mL 40/2 2500 lux Round 1						
	PI	SI	PSI	BPSI	PO	SO	PSO	BPSO	CI	PI	SI	CPI	CSI	PSI	CPSI
TH_2	1.567	1.074	1.328	1.408	1.159	0.918	0.955	1.046	2.88	1.54	1.81	2.56	1.89	2.13	1.83
H_2P	0.729	0.499	0.618	0.655	0.539	0.427	0.444	0.486	0.358	0.143	0.225	0.238	0.176	0.198	0.17
H_2Y	1.749	1.199	1.483	1.572	1.293	1.024	1.065	1.168	3.218	1.718	2.024	2.858	2.111	2.381	2.043
SCE	43.723	29.97	37.064	39.294	32.333	25.605	26.635	29.19	80.45	42.938	50.611	71.46	52.781	59.524	51.076
pH	7.55	7.35	7.15	7.33	8.3	7.79	7.25	7.92	6.94	7.34	7	7.05	7.03	7.02	7.16

	150 mL 40/2 2500 lux Round 2							150 mL 40/2 4000 lux Round 1							150 mL 40/2 4000 lux Round 2						
	CI	PI	SI	CPI	CSI	PSI	CPSI	CI	PI	SI	CPI	CSI	PSI	CPSI	CI	PI	SI	CPI	CSI	PSI	CPSI
TH_2	1.87	0.36	1.29	1.33	1.19	1.24	0.89	2.5	2.15	1.84	2.14	2.11	1.53	2.15	2.54	2.08	2.05	2.53	2.32	1.02	2.38
H_2P	0.174	0.045	0.16	0.124	0.111	0.115	0.082	0.465	0.4	0.343	0.398	0.392	0.284	0.4	0.363	0.298	0.294	0.362	0.332	0.146	0.34
H_2Y	2.086	0.403	1.438	1.485	1.33	1.383	0.989	2.79	2.4	2.055	2.39	2.353	1.702	2.4	2.83	2.322	2.291	2.821	2.592	1.141	2.654
SCE	52.161	10.076	35.962	37.125	33.25	34.567	24.724	69.754	59.989	51.386	59.756	58.826	42.55	59.989	70.762	58.051	57.276	70.53	64.794	28.522	66.344
pH	6.86	7.16	6.83	6.91	6.89	6.85	6.97	7.03	7.05	7.11	7.07	7.02	7.11	7.08	7.07	7.08	7.15	7.1	7.07	7.18	7.13

Table 3.10.1 continued.

	150 mL 40/2 Outdoor Round 1							150 mL 40/2 Outdoor Round 2						150 mL 60/2 2500 lux Round 1							
	CO	PO	SO	CPO	CSO	PSO	CPSO	CO	PO	SO	CPO	CSO	PSO	CPSO	CI	PI	SI	CPI	CSI	PSI	CPSI
TH_2	3.28	1.83	1.74	1.98	1.95	2.01	1.95	2.17	0.55	0.79	1.24	1.66	1.19	0.97	2.24	2.12	1.64	2.28	1.88	1.56	1.73
H_2P	0.406	0.171	0.162	0.245	0.242	0.249	0.241	0.202	0.051	0.073	0.116	0.154	0.111	0.088	0.417	0.394	0.305	0.423	0.35	0.29	0.321
H_2Y	3.655	2.046	1.944	2.207	2.179	2.241	2.173	2.421	0.617	0.88	1.389	1.851	1.327	1.082	1.668	1.575	1.221	1.693	1.401	1.159	1.283
SCE	91.378	51.153	48.596	55.184	54.486	56.036	54.331	74.095	24.182	41.543	51.773	48.828	39.76	26.429	41.698	39.373	30.537	42.318	35.032	28.987	32.087
pH	6.87	7.23	6.98	6.97	6.8	6.91	7.1	6.69	7.05	6.82	6.96	6.63	6.75	6.93	7.14	7.14	7.29	7.19	7.21	7.27	7.24
	150 mL 60/2 2500 lux Round 2							150 mL 60/2 4000 lux Round1						150 mL 60/2 4000 lux Round 2							
	CI	PI	SI	CPI	CSI	PSI	CPSI	CI	PI	SI	CPI	CSI	PSI	CPSI	CI	PI	SI	CPI	CSI	PSI	CPSI
TH_2	3.46	3.54	3.16	3.36	3.46	3.33	3.01	3.25	3.64	3.47	3.57	3.28	3.49	2.88	2.31	2.26	1.94	2.45	2.04	1.74	2.34
H_2P	0.358	0.384	0.336	0.364	0.37	0.359	0.321	0.403	0.451	0.43	0.443	0.407	0.433	0.357	0.286	0.28	0.241	0.304	0.253	0.216	0.29
H_2Y	2.433	2.612	2.282	2.476	2.513	2.443	2.185	2.418	2.708	2.582	2.656	2.44	2.597	2.143	1.719	1.682	1.443	1.823	1.518	1.295	1.741
SCE	60.816	65.311	57.044	61.901	62.831	61.074	54.615	60.454	67.708	64.546	66.406	61.012	64.918	53.571	42.969	42.039	36.086	45.573	37.946	32.366	43.527
pH	7.16	7.17	7.32	7.23	7.24	7.3	7.28	6.94	7.14	7.13	7.13	7.01	7.15	7.02	7.07	7.08	7.15	7.1	7.07	7.18	7.13

CHAPTER 4

CONCLUSION

In this study, biological hydrogen production was achieved by agar immobilized different PNSB strains on acetate in photobioreactors. Different bioreactors having volumes of 1.4 L, 300 mL and 150 mL have been tested. The photobioreactors were operated anaerobically indoor (continuous illumination) with different light intensities, such as 2500 lux and 4000 lux, and outdoors for 15-25 days using a defined H_2 production medium with different substrate concentrations, such as 40 mM Acetate + 2 mM Glutamate and 60 mM Acetate + 2 mM Glutamate. PNSB strains were tested as single, and double and triple co-cultures in an immobilized system.

The *R. capsulatus* (YO3) single culture was superior to all other groups in the experiments on 40 mM acetic acid concentration and the *Rp. palustris* (DSMZ 127) single culture was superior to all other groups in the experiments on 60 mM acetic acid concentration. However, both were observed to underperform in the presence of other strains in an immobilized setting. Still, their di-cultures have exhibited comparable performances to the better performing constituent species as single-cultures, hence the di-culture of *R. capsulatus* (YO3) and *Rp. palustris* (DSMZ 127) may provide the advantage of high hydrogen production performance both under high and low acetic acid concentration. Therefore, although synergistic benefits of immobilized co-cultivation were not observed, in means of obtaining higher hydrogen production performances, the di-culture of *R. capsulatus* (YO3) and *Rp. palustris* (DSMZ 127) may provide optimal hydrogen production performances under varying conditions, hence could be regarded as an advantageous co-cultivation couple of PNSBs for hydrogen production. Still, an increase in hydrogen production was not observed for immobilized and co-cultivated PNSB with respect to the single-cultures. The immobilized setup has a fixed bacterial composition due to arrested

growth. This lack of dynamism may be responsible for the inability to observe the concerted benefits of a co-cultivated suspended bacterial culture. However, the immobilized setting has its own advantages, such as avoiding bacterial washout, hence the ability to utilize wastes and eliminate lag phases, since the cultivation starts with fully grown bacteria and the ability to reuse the bacteria. The new reactor system design shows benefits in optimal sunlight utilization throughout the day in outdoor experiments, removes the requirement of repositioning the reactors during the day and the year for maximal illumination, hence decreasing the complication level for outdoor setups, and is promising. However, the contact area of the agar-bacteria mixture and the medium was limited, since one side of the agar-bacteria mixture was directly in contact with the reactor. Increasing the contact area of the agar-bacteria mixture and the medium might result in higher hydrogen production performances. Further studies may investigate techniques for preparing bacteria-agar mixtures not in contact with the reactor walls, but with a margin between the bacteria-agar mixture and the reactor. One technique would be, firstly pouring and solidifying a water-soluble spacer inside the reactors, then pouring and solidifying the bacteria-agar mixture inside the spacer, and then solving and eliminating the spacer from the reactor, prior to starting hydrogen production experiments. It is also necessary to research various temperatures, light intensities, and substrate concentrations to better understand how hydrogen is produced in immobilized co-cultures and standardize hydrogen yields and productivity. Studies regarding the mechanistic understanding of bacterial interactions in an immobilized setting are required. Also, investigating hydrogen production by immobilized PNS bacteria in a bio-film format would be beneficial since the immobilization type may be applied to larger-scale production setups and reactors.

The experiments performed with 1.4 L reactors did not yield desirable hydrogen production performances, hence were abandoned after the first round of the first run. The experiments performed with 300 mL reactors have exhibited an improvement during the first run, however the improvement was not on the desirable level, hence the first run was shut down after the second round. The experimental procedure was

optimized during the second run; hence the second run was continued for five rounds. During those five rounds, hydrogen production performances were comparable, and although were higher than the first round, were not on the desired level. Hence the experimentations with the 150 mL reactors were begun. Since the 5 rounds of the second run using the 300 mL reactors yielded comparable results, the experiments with the 150 mL reactors were continued for one run and two rounds. Future studies may be performed with more runs and more rounds.

Additionally, future studies may integrate lateral in-reactor bacteria-culture solidification on a larger scale, using larger reactor volumes.

For outdoor experiments, the hydrogen production performances during other seasons throughout the year may be investigated.

In summary, further investigation is required to increase the process's efficiency and feasibility.

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APPENDICES

A. COMPOSITIONS OF MEDIA, VITAMINS, TRACE ELEMENT AND Fe-CITRATE SOLUTIONS

Table A.1 The composition of 1 liter of growth and hydrogen production medium

Medium Composition	Growth Medium	Hydrogen Production Medium
KH_2PO_4	3 g	3 g
$MgSO_4.7H_2O$	0.5 g	0.5 g
$CaCl_2.2H_2O$	0.05 g	0.05 g
Glacial Acetic Acid	1.15 ml	2.29 ml
Na-Glutamate	1.85 g	0.36 g
Vitamin Solution	0.1 ml	0.1 ml
Trace Element Solution	0.1 ml	0.1 ml
Fe-Citrate	0.5 ml	0.5 ml

Table A.2 The composition of 100 mL of vitamin solution (10 x)

Composition	Amount
Thiamine chloride hydrochloride	500 mg
Niacin (Nicotinic Acid)	500 mg
D+ Biotin	15 mg

Table A.3 The composition of 100 mL of trace element solution (10 x)

Composition	Amount
$ZnCl_2$	70 mg
$MnCl_24.H_2O$	100 mg
H_3BO_3	60 mg
$CoCl_2.6H_2O$	200 mg
$CuCl_2.2H_2O$	20 mg
$NiCl_2.6H_2O$	20 mg
$Na_2MoO_4.2\ H_2O$	40 mg
HCl (25% V/V)	1 mg

The composition of 100 mL of Fe-citrate solution (50 x):

5 g Fe-citrate was dissolved in 100 mL distilled water and autoclaved for sterilization.

B. ORGANIC ACID ANALYSIS

B.1 Sample Acetate HPLC Chromatogram

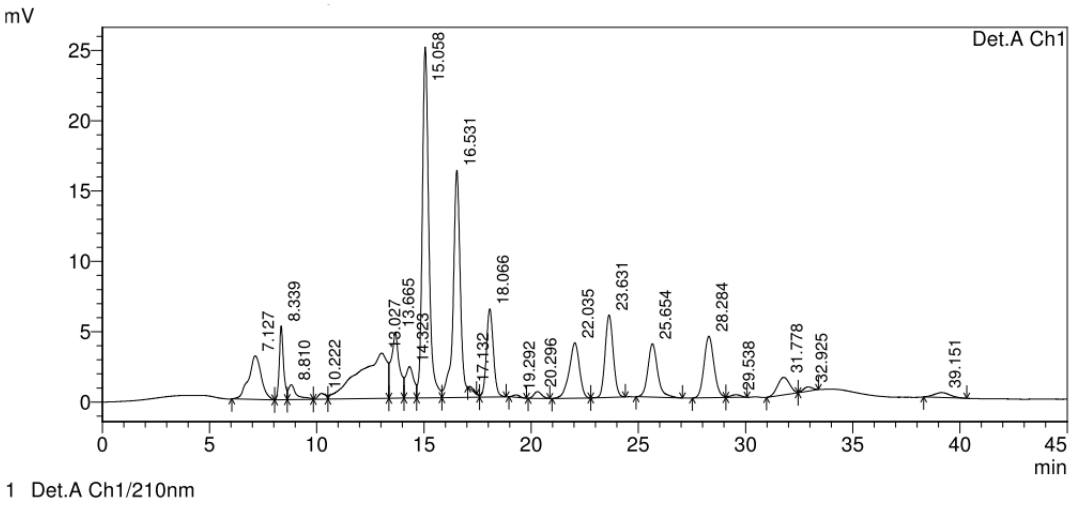


Figure B.1 Sample HPLC organic acid chromatogram (Acetate peak in 25.654 min, Shimadzu Agilent 10A series HPLC, UV 210 nm detector).

B.2 Acetate HPLC Calibration Curve

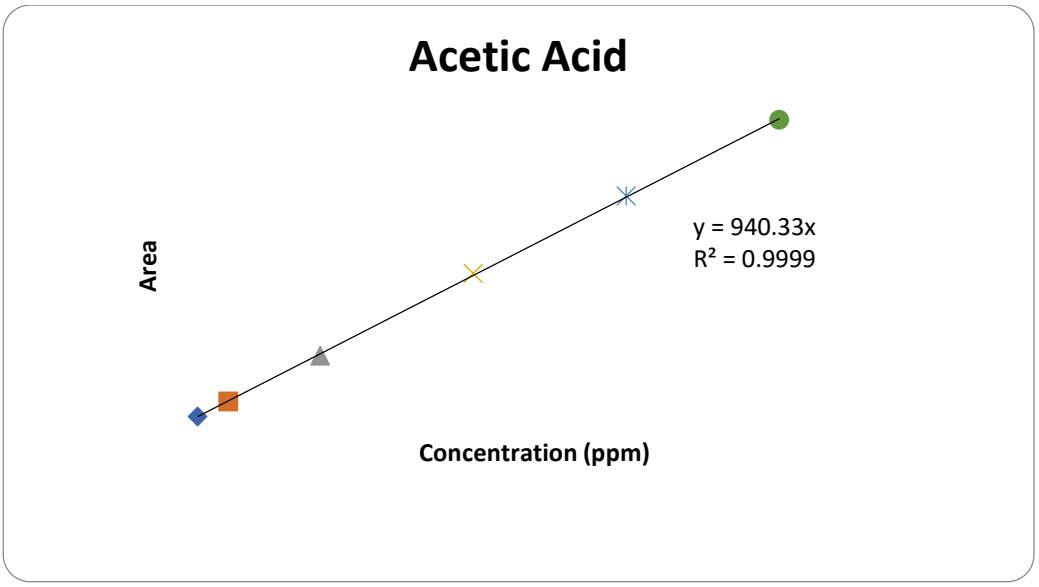
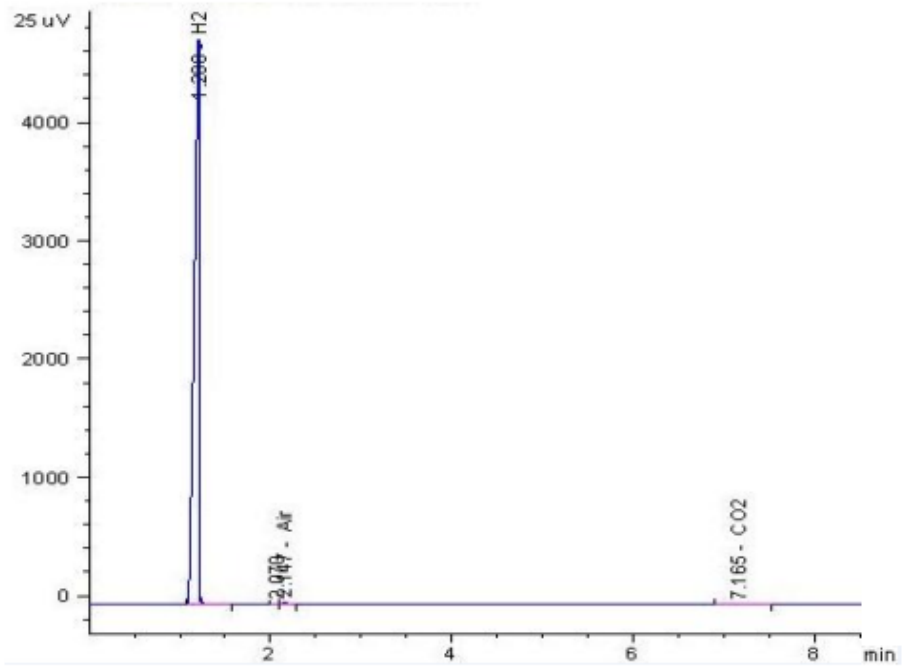


Figure B.2 Sample curve of sample acetate.

C. SAMPLE GAS CHROMATOGRAM



RetTime [min]	Type	Area [25 uV*s]	Amt/Area	Norm %	Grp	Name
1.208	BB	1.78157e4	5.36760e-3	92.790983		H2
2.147	VB	104.16668	4.30476e-2	4.351104		Air
7.165	BB	52.88443	5.56929e-2	2.857913		CO2

Figure C.1 Sample gas chromatogram (Agilent Technologies 6890 N gas chromatography) (Androga, 2009).

D. HYDROGEN YIELD CALCULATION

$$\text{Hydrogen Yield} = \frac{\text{Mass of hydrogen produced (g)}}{\text{Mass of substrates utilized}}$$

A sample substrate conversion efficiency (yield) calculation for the 40 mM/2 mM Ac/Glu fed *R. capsulatus* hup- (YO3) + *R. palustris* (DSM 127) + *R. sphaeroides* O.U.001 (DSM 5864).

$$= \frac{2 \times 1.00 \text{ (g / mol)} \times 272.8 \text{ (mL)} \times 4.02 \times 10^{-5} \text{ (mol / mL)}}{0.10 \text{ (L)} \times 0.04 \text{ (mol)} \times 60.05 \text{ (g / mol)}}$$

E. SUBSTRATE CONVERSION EFFICIENCY CALCULATION

$$\text{Substrate conversion efficiency} = \frac{\text{Actual moles of } H_2 \text{ produced}}{\text{Theoretical moles of } H_2 \text{ produced}} \times 100$$

$$= \frac{\text{moles of } H_2 \text{ produced}}{4 \times \text{moles of Acetate Utilized}}$$

A sample substrate conversion efficiency (yield) calculation for the 40 mM/2 mM Ac/Glu fed *R. capsulatus* hup- (YO3) + *R. palustris* (DSM 127) + *R. sphaeroides* O.U.001 (DSM 5864).

$$= \frac{0.27 \text{ L} \times 4.02 \times 10^{-5} \text{ g/mol}}{0.105 \text{ L} \times 4.40 \text{ g/mol}} \times 100 = 65.28\%$$

F. HYDROGEN PRODUCTIVITY CALCULATION

Calculation of hydrogen productivity

A sample hydrogen productivity calculation for the 40 mM/2 mM Ac/Glu fed *R. capsulatus* hup- (YO3) + *R. palustris* (DSM 127) + *R. sphaeroides* O.U.001 (DSM 5864).

t = Duration of hydrogen production (hour) = 96 h
v = Volume of culture = 0.15 l
V_{H2} = Produced hydrogen = 10.97 mmol

Hydrogen productivity (mmol $H_2/L_c.h$)
= 10.97 mmol / (0.15 L x 96 h) = 0.76 mmol / L x h

G. ORGANIC ACID VARIATION PROFILES

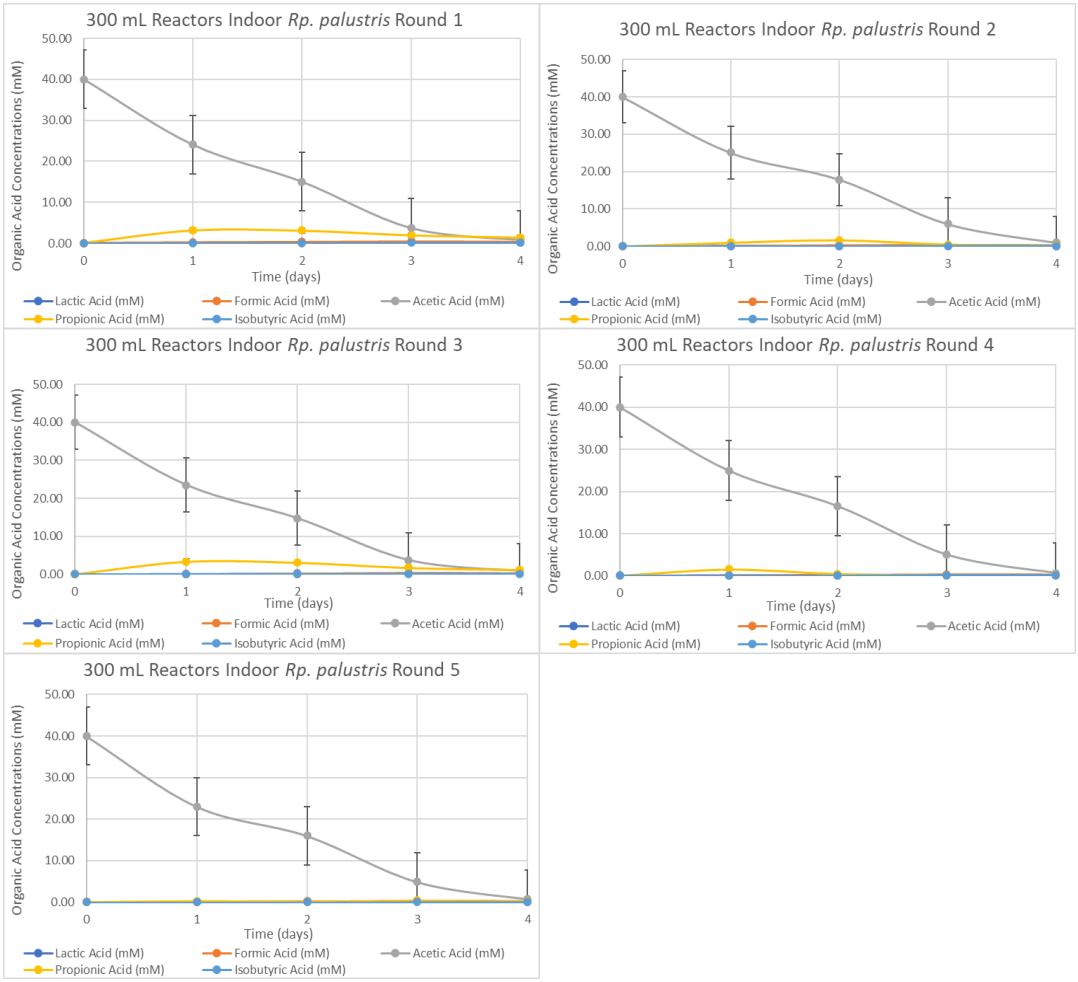


Figure G.1 Organic acid variation profiles for 300 mL single cultures of *Rp. palustris* (DSMZ 127) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

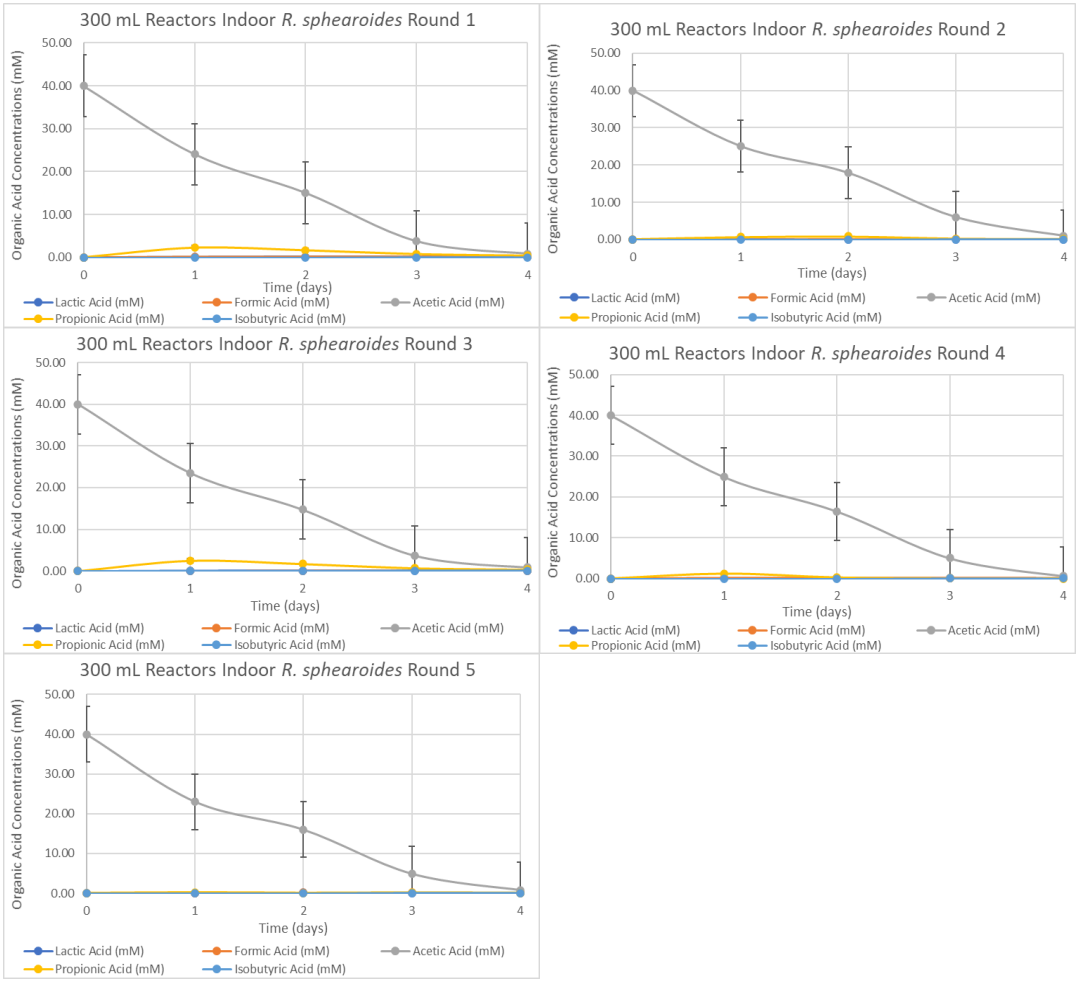


Figure G.2 Organic acid variation profiles for 300 mL single cultures of *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

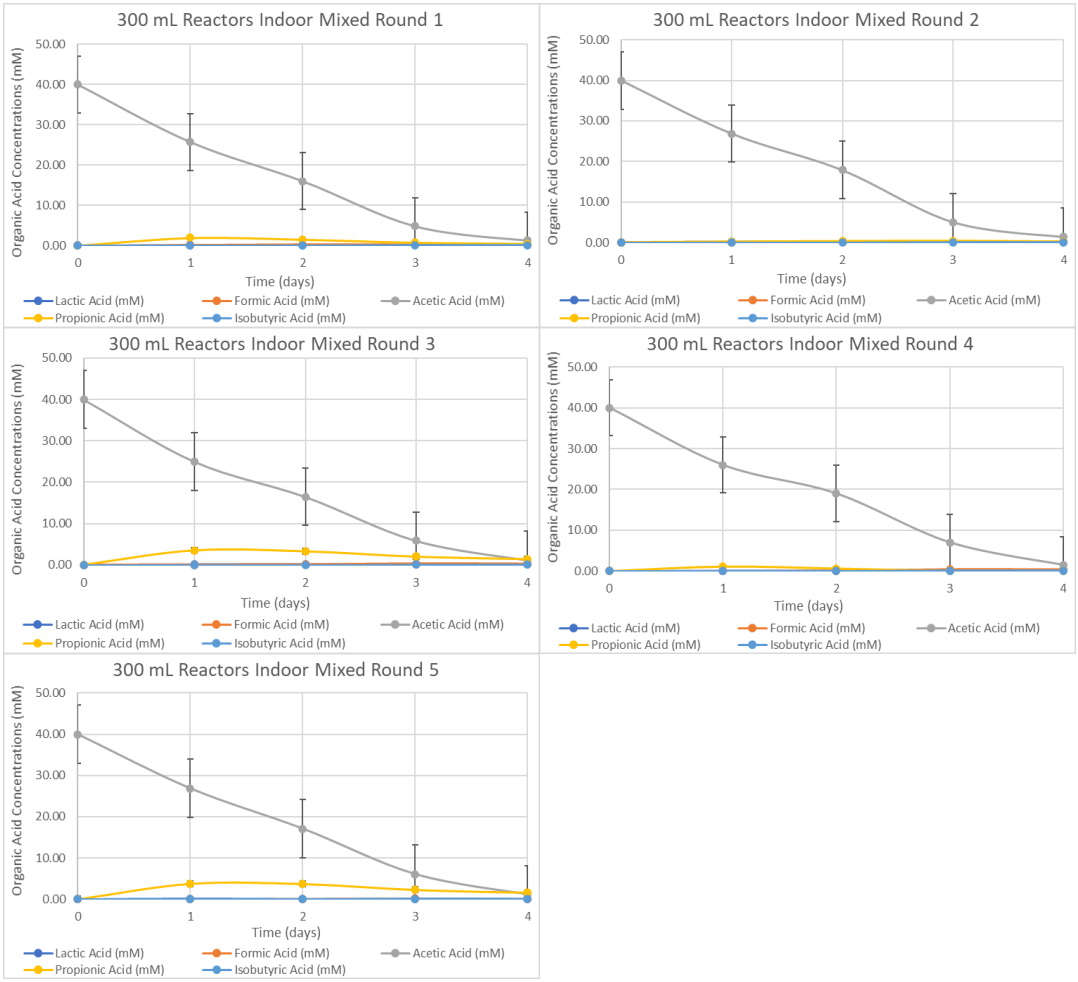


Figure G.3 Organic acid variation profiles for 300 mL mixed co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

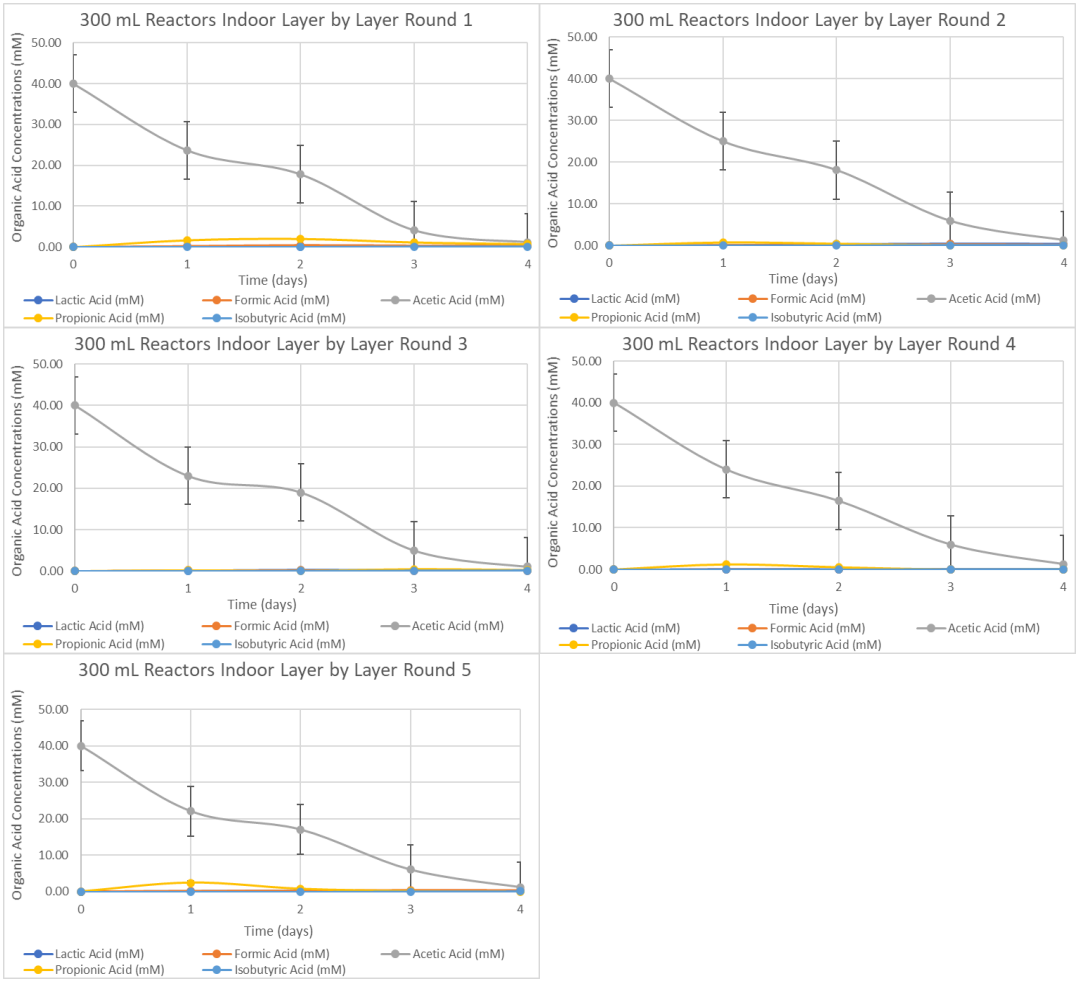


Figure G.4 Organic acid variation profiles for 300 mL bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux.

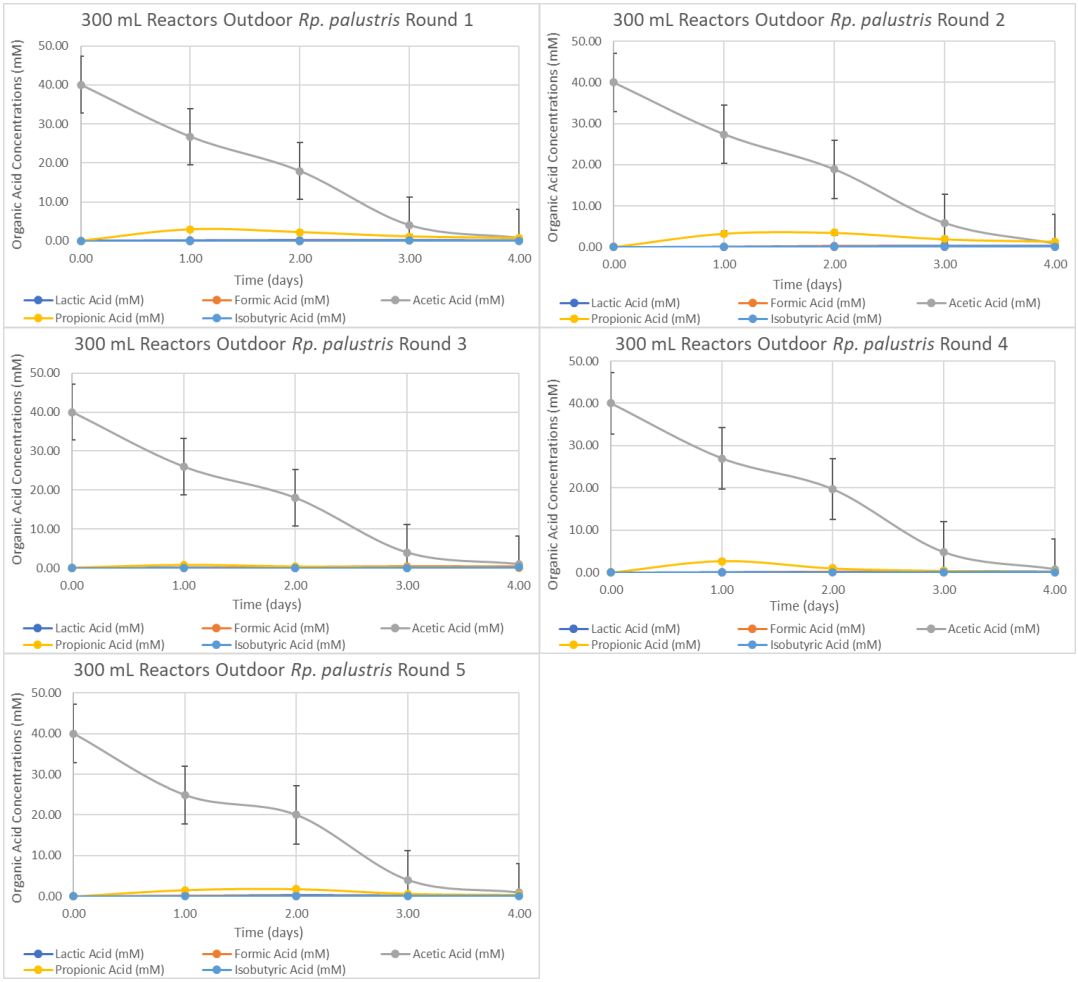


Figure G.5 Organic acid variation profiles for 300 mL single cultures of *Rp. palustris* (DSMZ 127) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight.

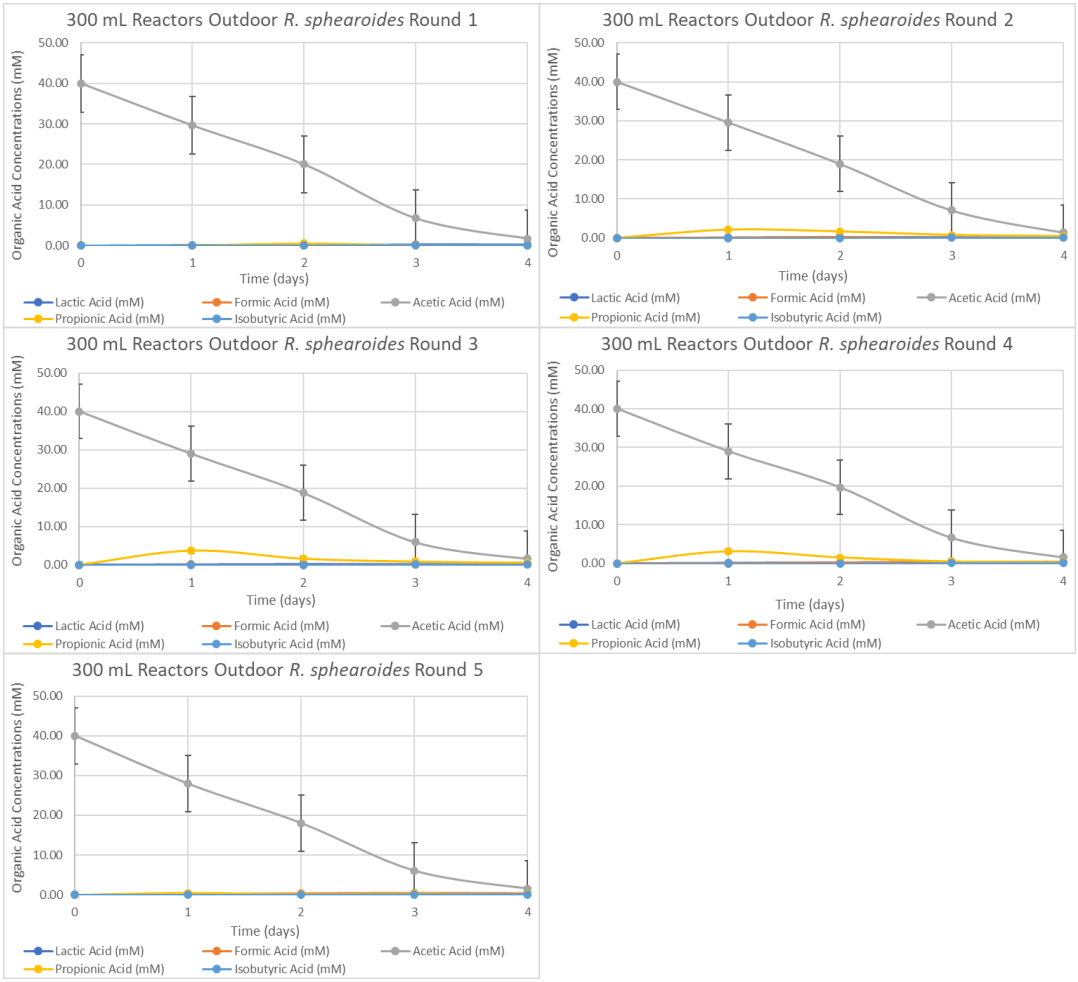


Figure G.6 Organic acid variation profiles for 300 mL single cultures of *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight.

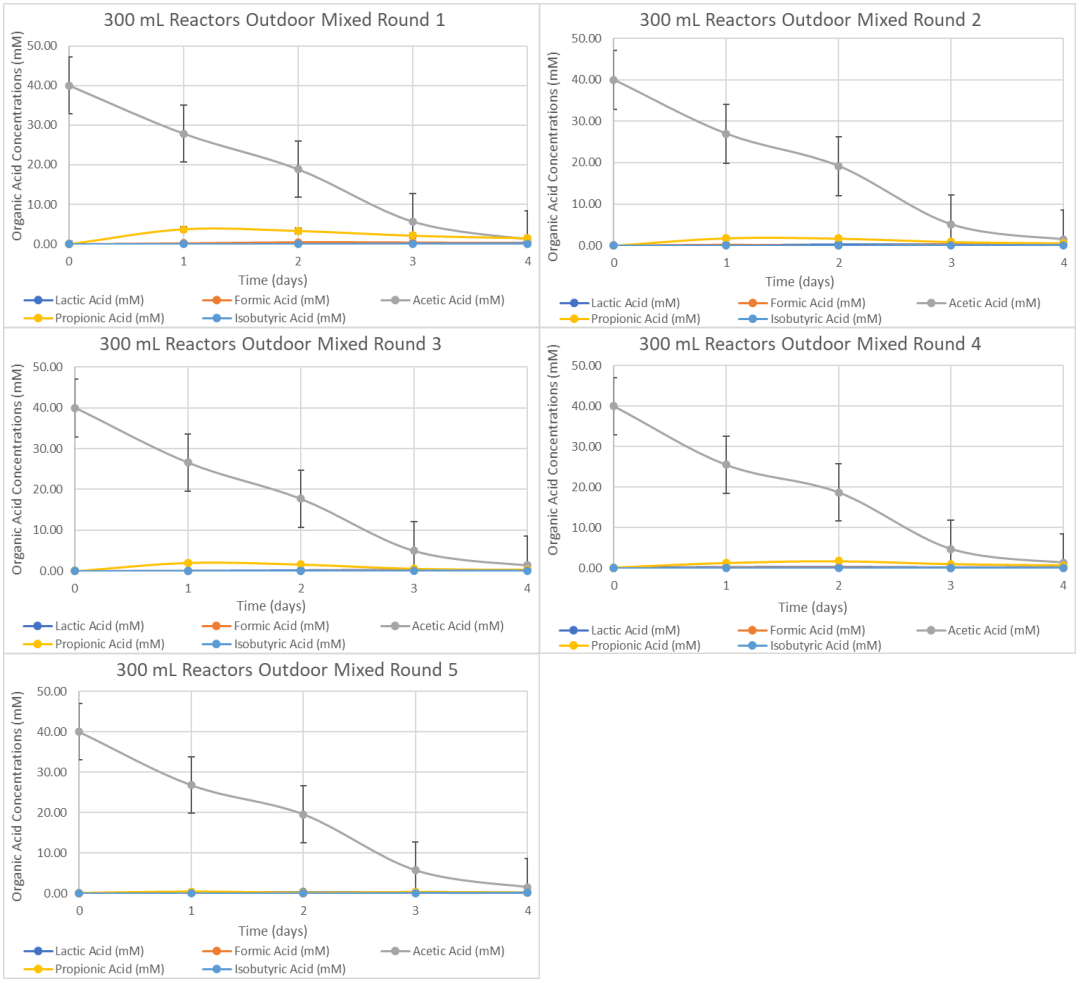


Figure G.7 Organic acid variation profiles for 300 mL mixed co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight.

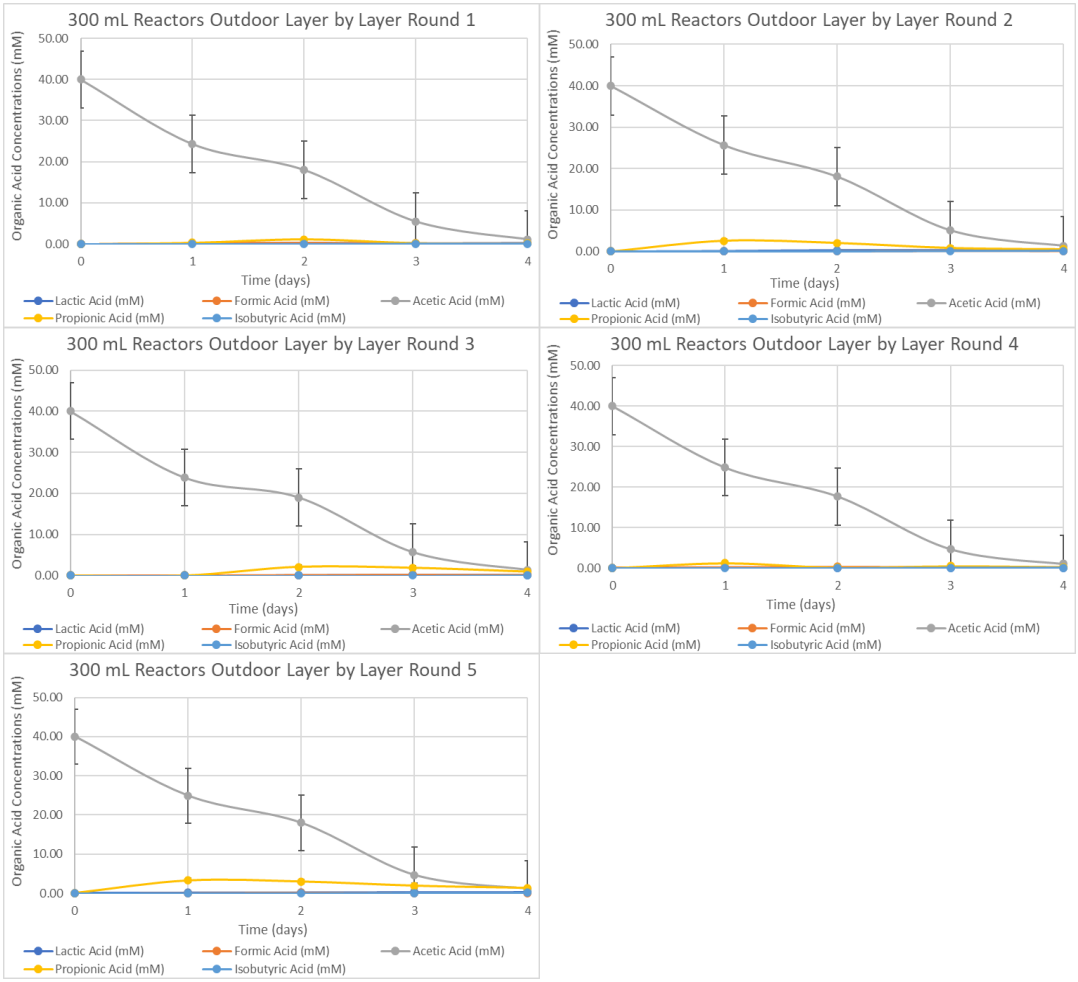


Figure G.8 Organic acid variation profiles for 300 mL bi-layer co-cultures of *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight.

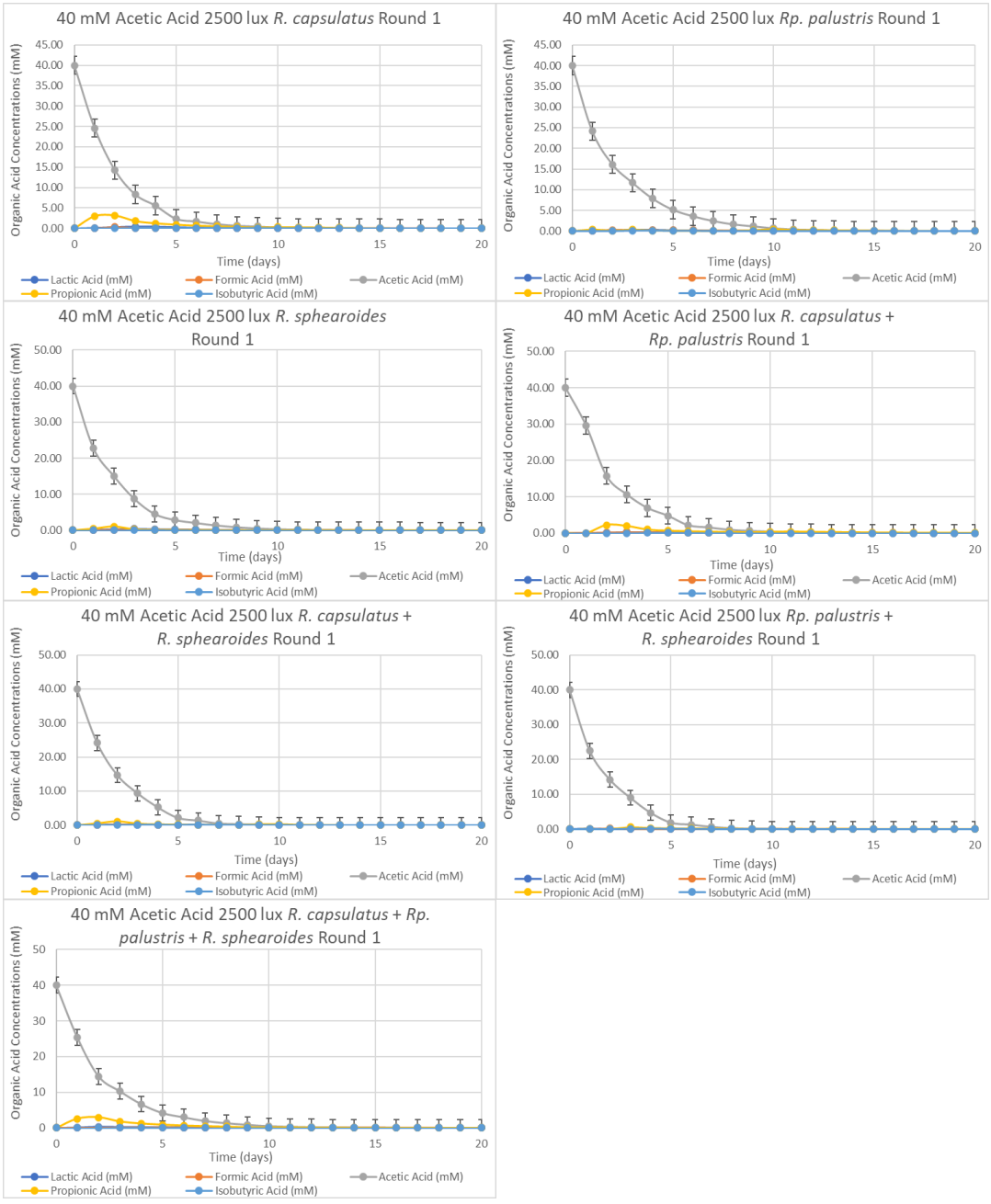


Figure G.9 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux. Round 1.

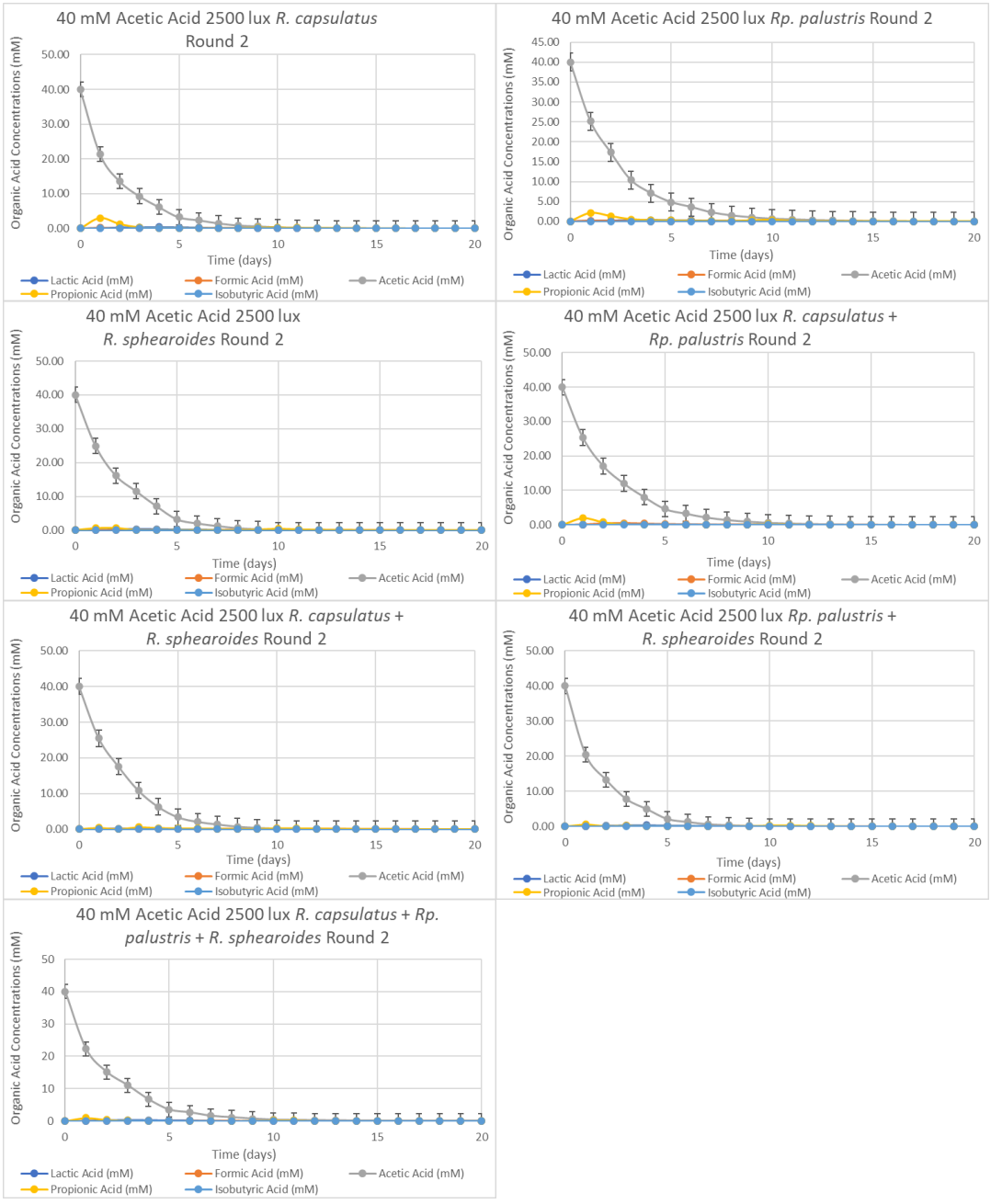


Figure G.10 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux. Round 2.

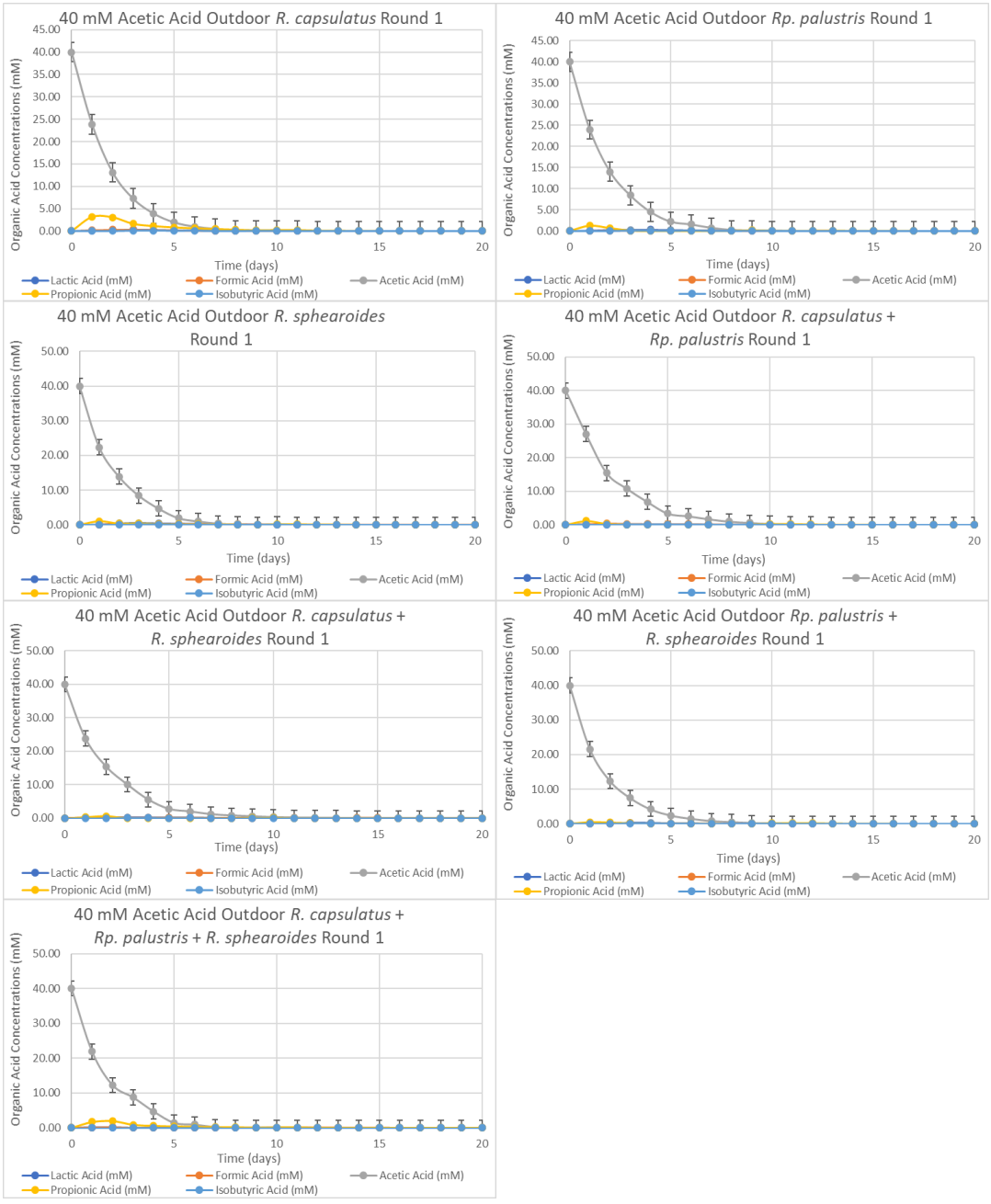


Figure G.11 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight. Round 1.

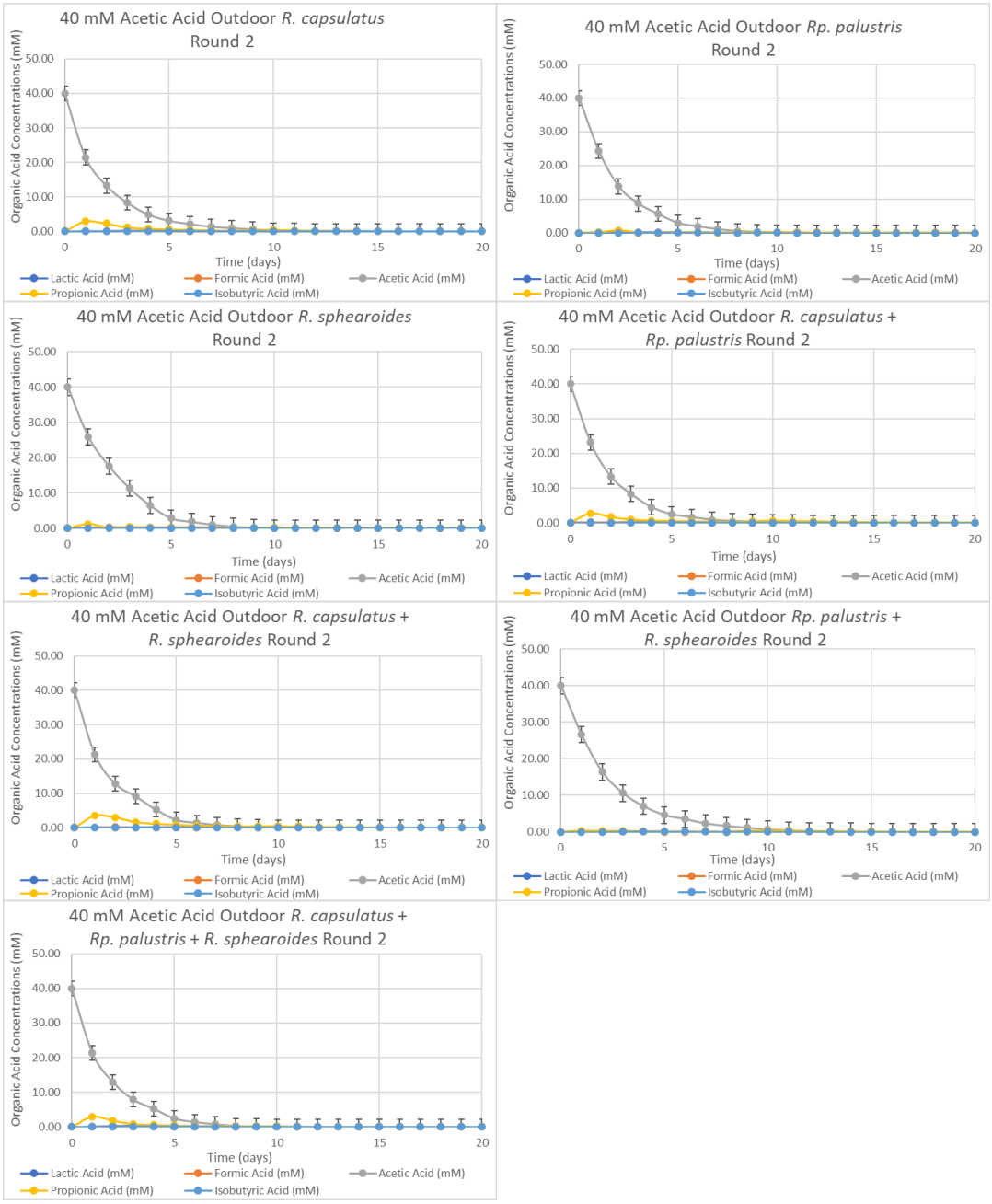


Figure G.12 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and sunlight. Round 2.

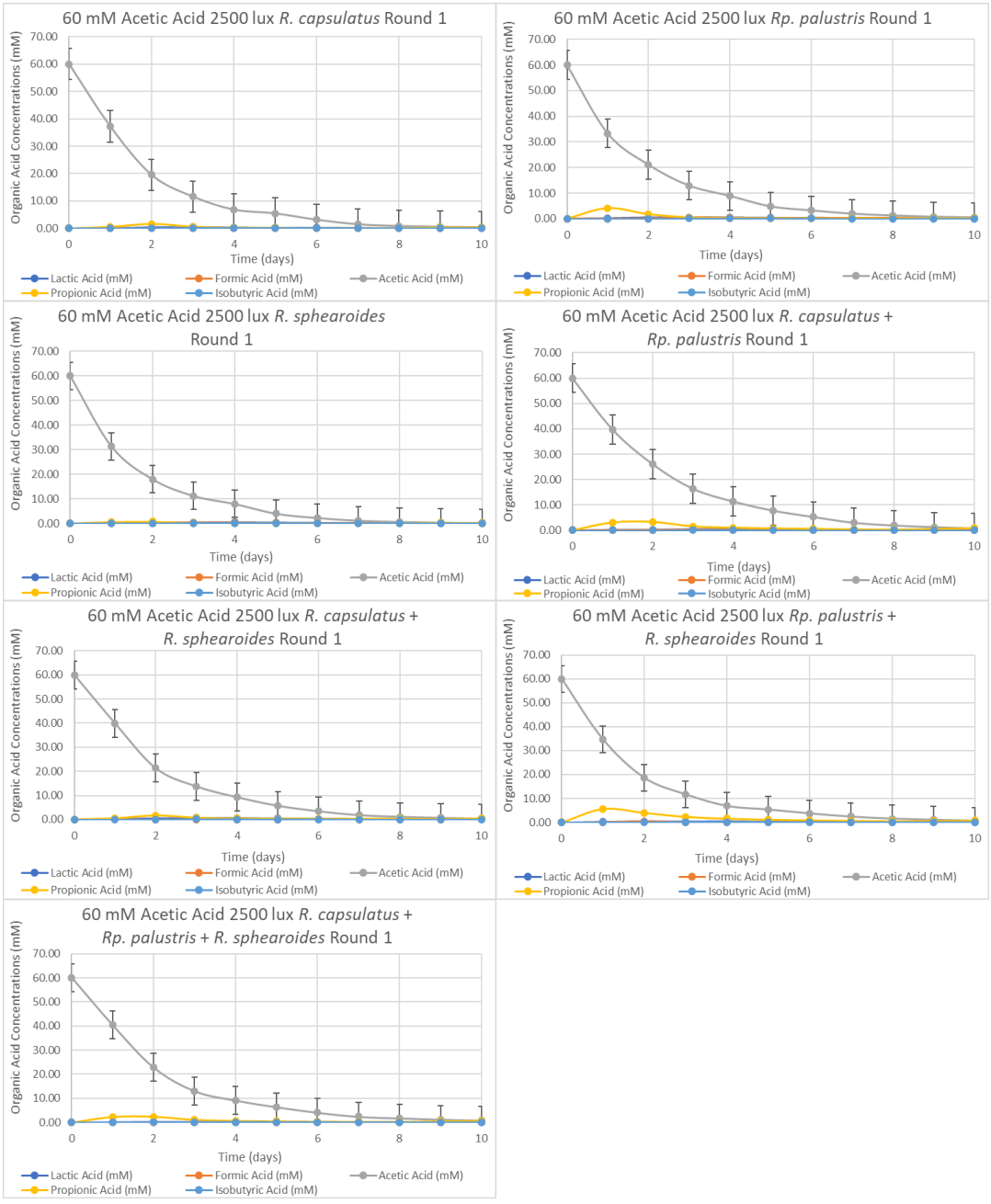


Figure G.13 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux. Round 1.

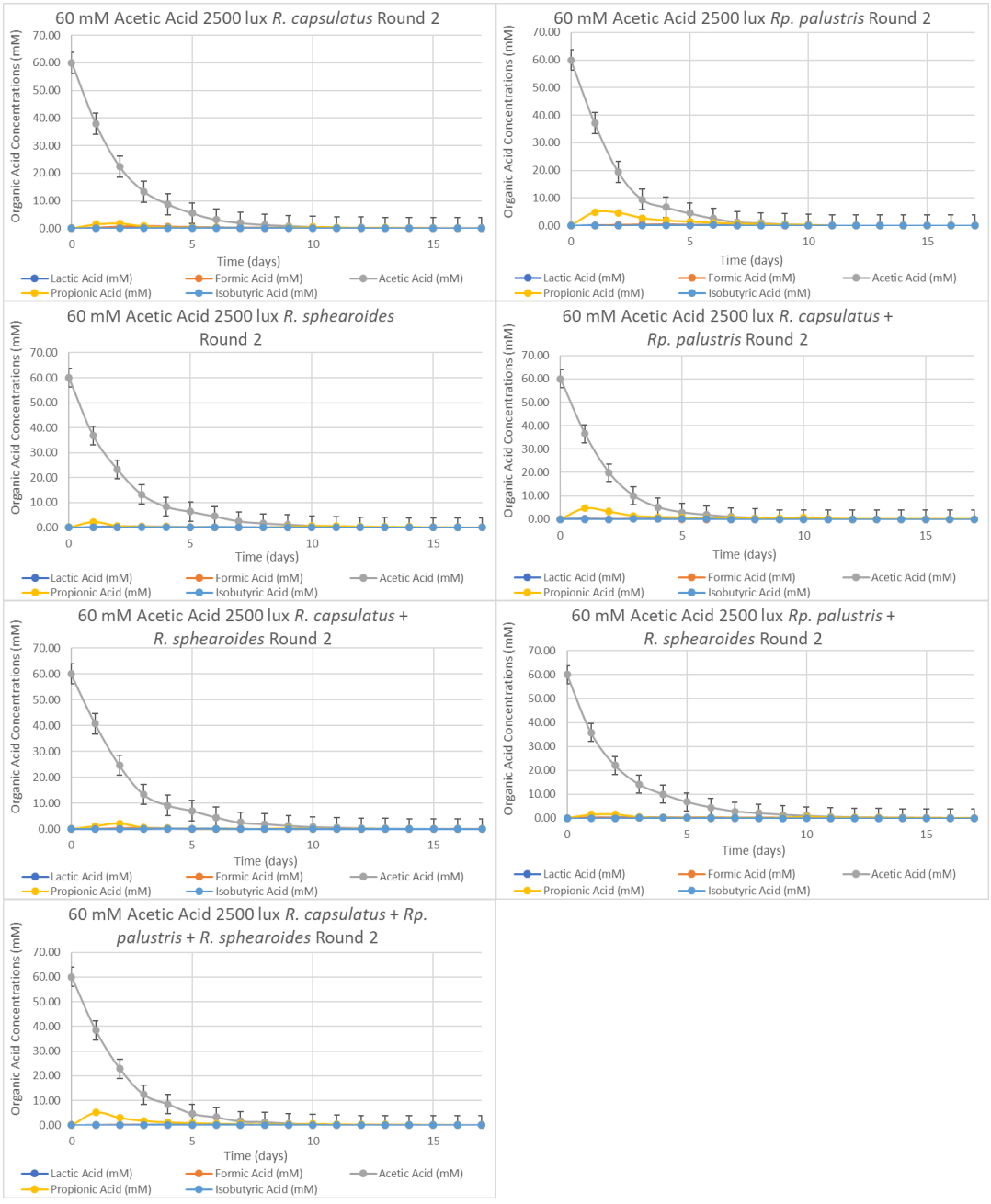


Figure G.14 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 2500 lux. Round 2.

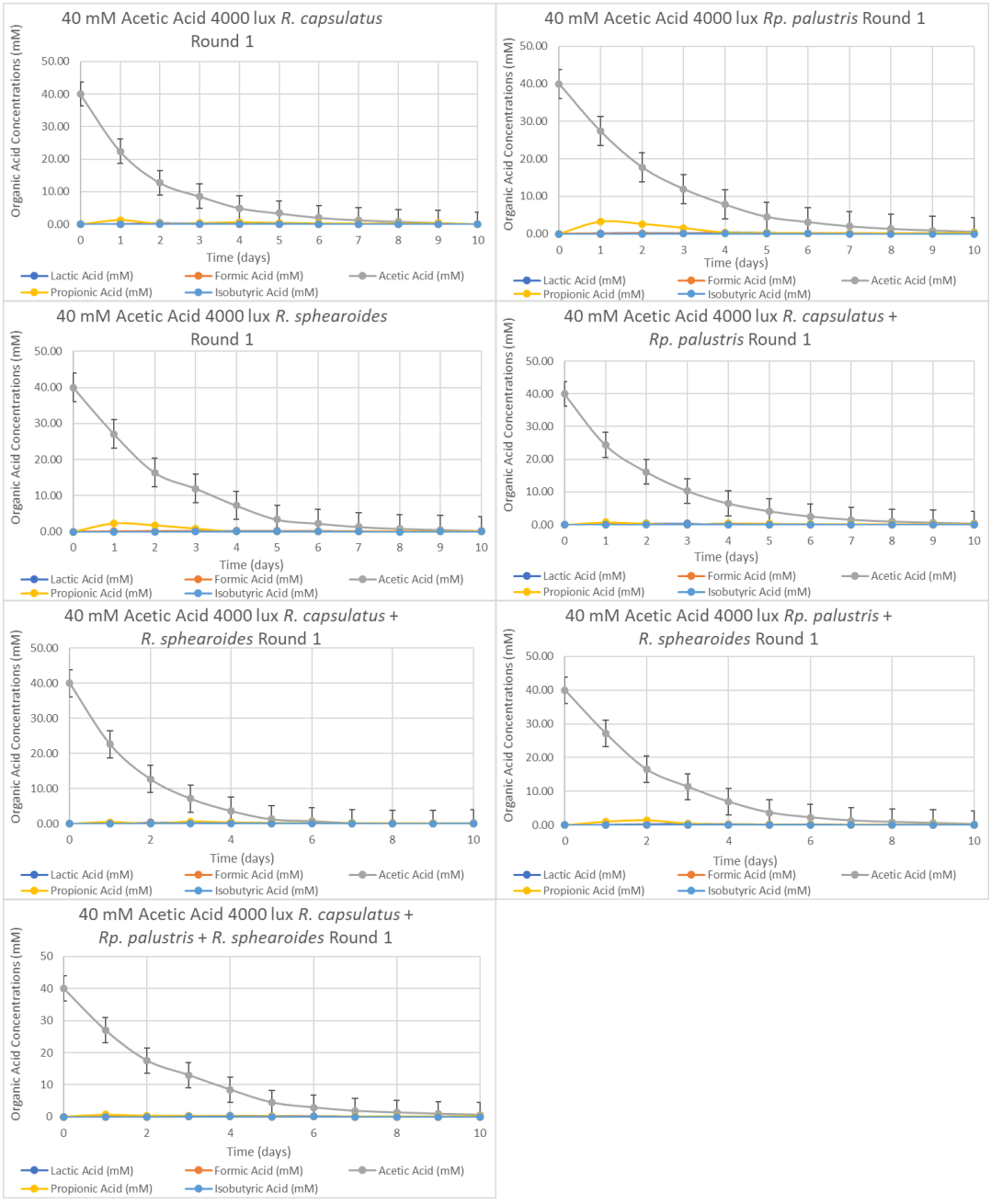


Figure G.15 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux. Round 1.

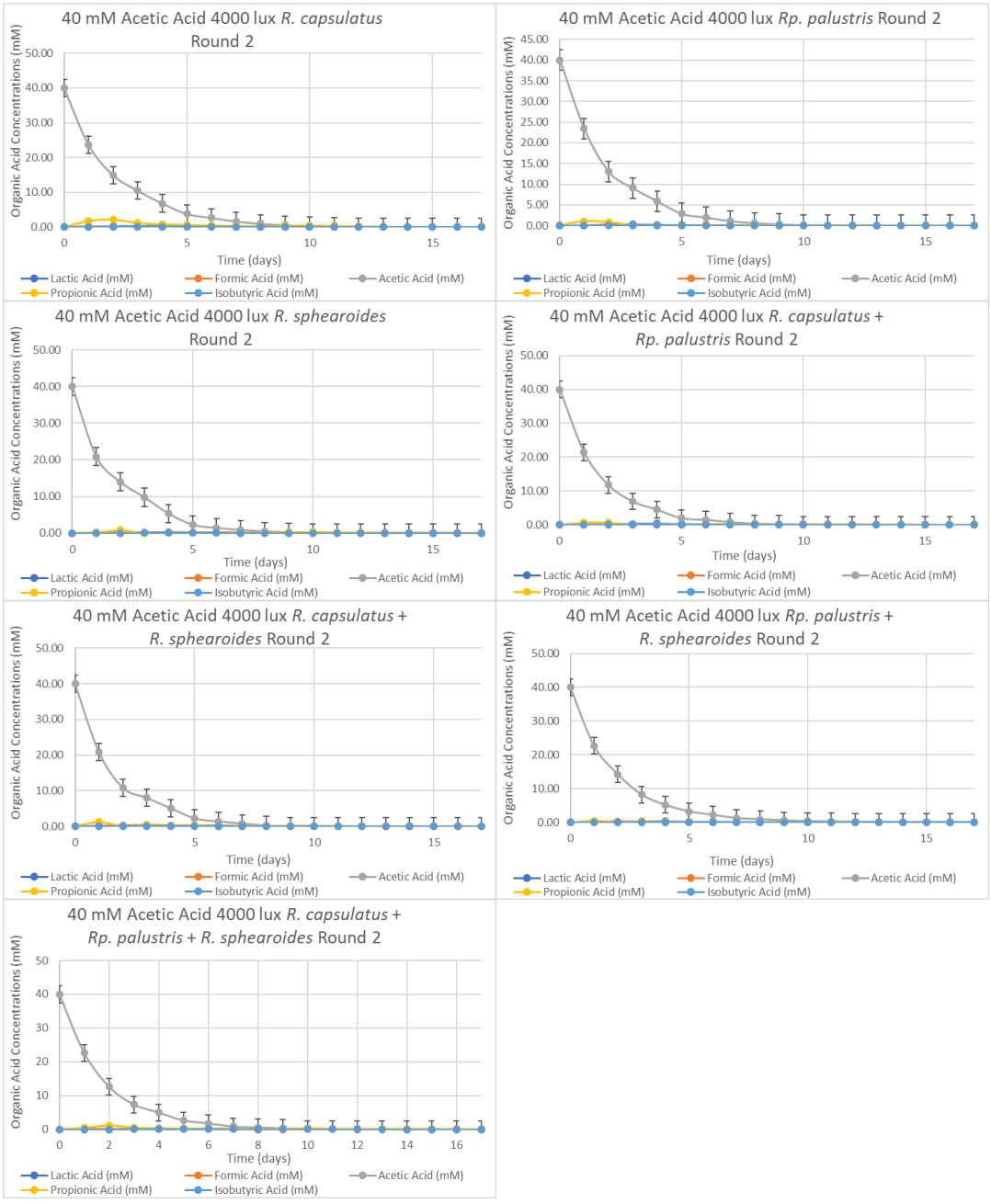


Figure G.16 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 40 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux. Round 2.

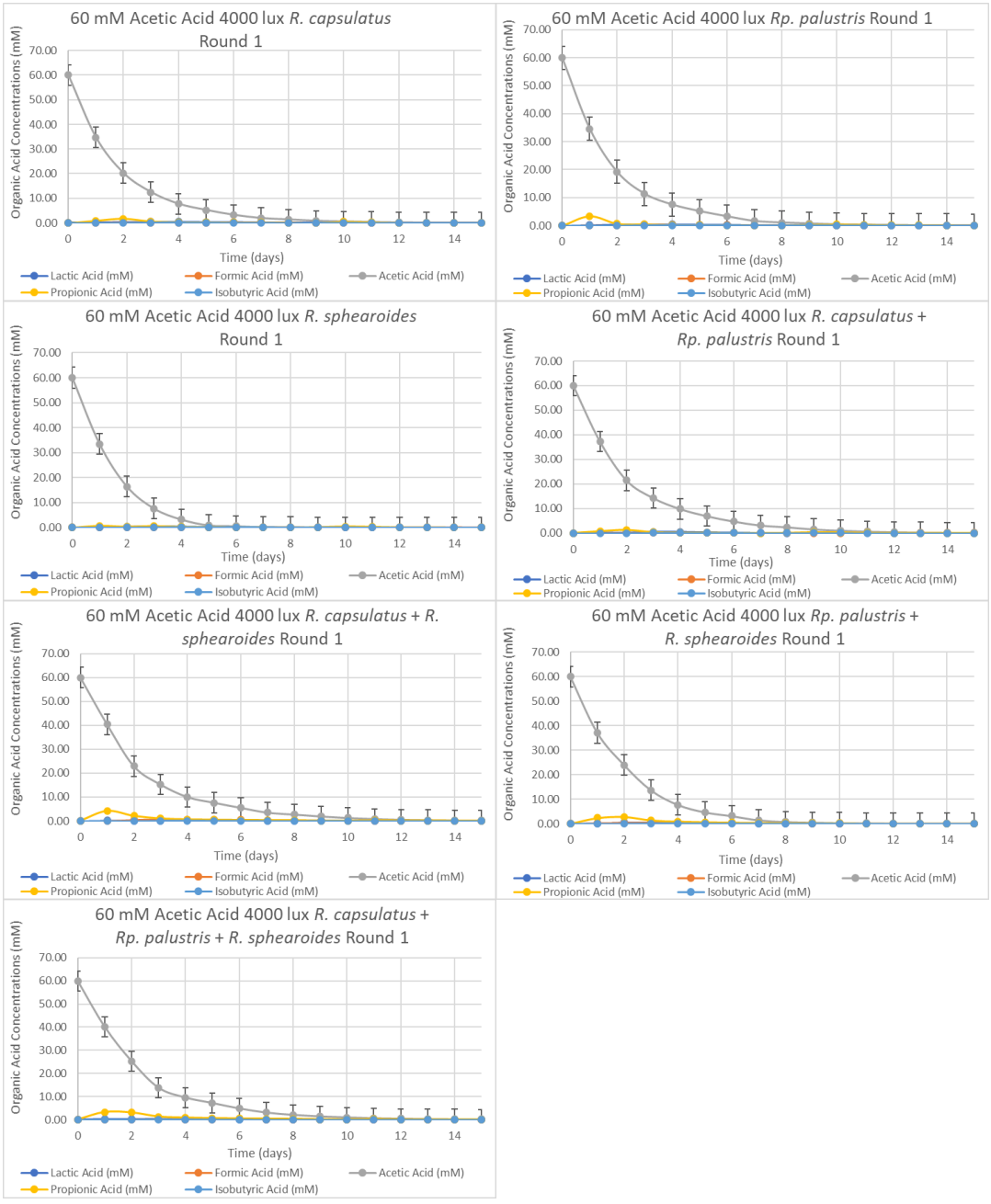


Figure G.17 acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux. Round 1.

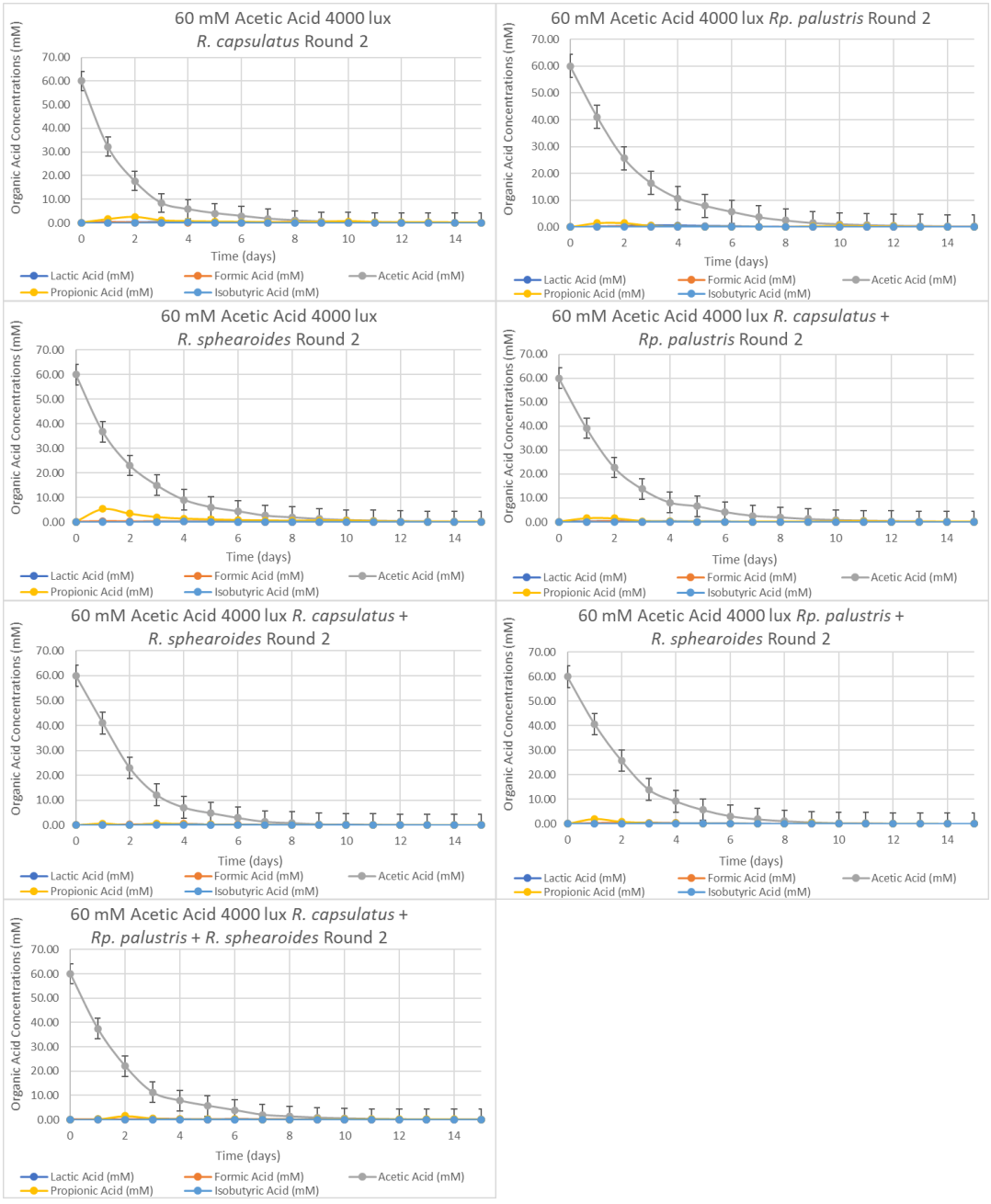


Figure G.18 Organic acid variation profiles for 150 mL single cultures and double and triple co-cultures of *R. capsulatus* hup- (YO3), *Rp. palustris* (DSMZ 127) and *R. sphaeroides* O.U.001 (DSMZ 5864) in an immobilized setting, cultivated on BP medium supplemented with 60 mM Acetate/2 mM Glutamate at 30 °C under deoxygenated atmosphere and uninterrupted illumination of 4000 lux. Round 2.

H. AUTHOR WITH HIS ADVISOR AND CO-ADVISOR

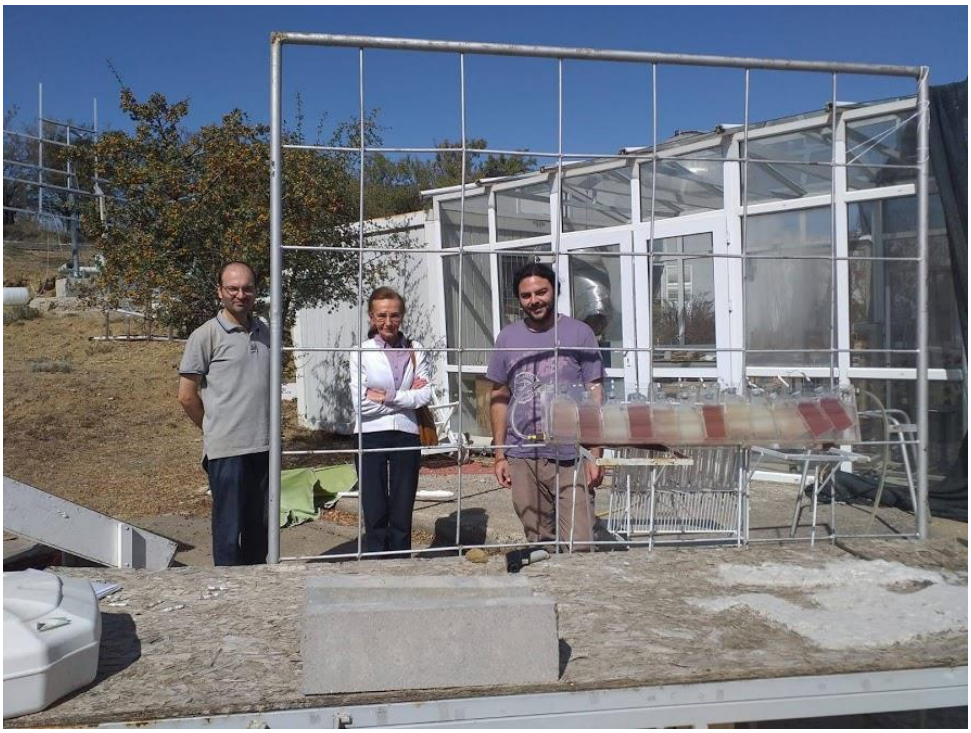


Figure H.1 Author with Advisor Prof. Dr. Meral Yücel and Co-Advisor Assoc. Prof. Dr. Harun Koku

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PUBLICATIONS

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CONFERENCES

4th International Hydrogen Technologies Congress (IHTEC-2019)

5th International Hydrogen Technologies Congress (IHTEC-2021)

EXTENDED ABSTRACTS

Biological Hydrogen Production by Using Co-Cultures of PNS Bacteria (IHTEC 2019)

Photofermentative Hydrogen Production by Agar Immobilized Co-Cultures of Purple Non-Sulfur Bacteria (IHTEC 2021)

PRESENTATIONS

Biological Hydrogen Production by Using Co-Cultures of PNS Bacteria (IHTEC 2019)

Photofermentative Hydrogen Production by Agar Immobilized Co-Cultures of Purple Non-Sulfur Bacteria (IHTEC 2021)